Novel Insights into the Molecular Pathogenesis of Gastric MALT Lymphoma

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SUMMARY

Gastric marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) represents a distinct class of extranodal lymphoma that evolves against a background of chronic inflammation induced by persistent infection with the bacterium *Helicobacter pylori*. In its early stages, MALT lymphoma is an antigen-dependent disease characterised by an indolent clinical course and in most cases is treatable by antibiotic eradication therapy alone. Low grade MALT lymphomas can eventually undergo high grade transformation to a more aggressive counterpart termed gastric diffuse large B-cell lymphoma (gDLBCL). At this stage the lymphomas grow autonomously and are refractory to *Helicobacter* eradication therapy. Little is known about the molecular mechanisms governing the development of low grade MALT lymphoma and its ultimate progression to gDLBCL. To improve our current understanding of the molecular pathogenesis of MALT lymphoma, a multidimensional approach was taken to investigate various aspects of this disease during the course of my PhD thesis.

Although a variety of circumstantial evidence has implicated an important role for antigen in MALT lymphomagenesis, the identity of such ligands recognised by the tumour cells remain unclear. To address this issue of antigen-receptor specificity, enzyme immunoassays were employed to systematically analyse the antigen binding profile of a comprehensive panel of human and murine recombinant MALT lymphoma-derived antibodies. The majority of tumour derived immunoglobulins were found to exhibit a broad reactivity profile in line with the definition of polyreactivity. In addition, using the BALB/c mouse model of *Helicobacter*-induced gastric MALT lymphoma, we demonstrated that explanted tumour cells proliferated in response to a variety of cognate antigens recognised by their polyreactive B-cell receptor. Our results therefore suggest that MALT lymphoma development may be facilitated by an array of local self- and foreign antigens, providing direct antigenic stimulation of the tumour cells via their B-cell receptor.

A characteristic feature of gastric MALT lymphomas is that they are typically infiltrated by large numbers of T-helper cells producing interleukin-4 (IL4) and other T-

helper cell type 2 cytokines. Therefore, an additional aim of this study was to elucidate the pathogenic role of the tumour-infiltrating T-cell population. Tumour cell proliferation was strongly enhanced by the presence of intratumoural $CD4^+$ T cells in a CD40/CD40L-independent manner. Another key finding was that a substantial fraction of tumour-infiltrating $CD4^+$ T cells were functional $CD25^+FoxP3^+$ regulatory T (Treg) cells. These cells were found to be recruited by the tumour cells themselves through secretion of the Treg-attracting chemokines CCL17 and CCL22. Interestingly, the depletion of $CD25^+$ T cells was as efficient as $CD4^+$ T-cell depletion in blocking tumour growth *ex vivo* and *in vivo*. Judging from our data, we propose that MALT lymphoma cells require at least two independent signals for proliferation. One signal is received by the functional surface-bound polyreactive immunoglobulin, while the other signal is delivered by tumour-infiltrating T cells, in particular by Tregs, which are likely to play a direct role in stimulating tumour growth.

To date, MALT lymphoma research has largely focused on altered expression of protein coding genes. However, recent evidence suggests that alterations of non-coding RNA, particularly microRNA (miRNA), also contribute to tumourigenesis. Using a genome-wide microarray-based approach, we defined the unique miRNA expression signature associated with the development and progression of this disease. A special focus was first laid upon miR-203 and its putative tumour suppressive function during the progression from reactive *Helicobacter*-specific gastritis to MALT lymphoma. We identified miR-203 to be significantly downregulated in MALT lymphoma tissue due to hypermethylation of the *miR-203* locus. The restoration of miR-203 expression in primary MALT lymphoma cells repressed the recently identified miR-203 target, ABL1, and blocked tumour cell proliferation. Finally, pharmacological inhibition of ABL1 activity by imatinib blocked MALT lymphoma cell proliferation *ex vivo* and effectively eradicated tumours *in vivo*. Collectively, our observations suggest that ABL1 plays an important role in MALT lymphoma cell biology and support a novel potential application of imatinib in the treatment of MALT lymphoma.

Our genome-wide survey further revealed a characteristic set of MYC-repressed miRNAs to be specifically downregulated in human gDLBCL compared to MALT

lymphoma and gastritis. Aberrant MYC expression indeed correlated with high grade transformation as evident from our tissue microarray analysis. The re-expression of a panel of selected MYC-associated miRNAs significantly reduced the proliferation of DLBCL cells. In particular, miR-34a was found to represent the most potent tumour suppressor in DLBCL cell lines. We could further attribute the tumour suppressive effects of miR-34a to dysregulation of its target FOXP1. Accordingly, FOXP1 overexpression was found to be strongly associated with gDLBCL and the transient knockdown of FOXP1 in DLBCL cell lines significantly impaired the proliferation of the tumour cells. Taken together, our findings elucidate a novel mechanism linking the aberrant expression of MYC and concomitant repression of miR-34a to FOXP1 deregulation in high grade transformation of MALT lymphoma.

ZUSAMMENFASSUNG

Gastrische Marginalzonen-B-Zell Lymphome des Schleimhaut-assoziierten lymphatischen Gewebes (MALT Lymphome) stellen eine eigene Klasse der extranodalen Lymphome dar, die sich nach chronischer Magenentzündung infolge persistierender Infektion mit dem bakteriellen Humanpathogen *Helicobacter pylori* bilden. Im Frühstadium zeichnen sich MALT Lymphome durch einen relativ indolenten klinischen Verlauf aus; in der Mehrheit der Fälle können die früh diagnostizierten Lymphome aufgrund ihrer Abhängigkeit von der *Helicobacter*-Infektion durch eine antibiotische Therapie geheilt werden. Niedrigmaligne MALT Lymphome können sich potentiell in hochmaligne, aggressive Lymphome entwickeln, welche dann als „grosszelliges B-Zelllymphom des Magens“ (gDLBCL) bezeichnet werden. In diesem Stadium sind die Lymphome antigenunabnängig und lassen sich durch *Helicobacter*-Eradikation nicht behandeln. Über die molekularen Mechanismen der hochgradigen Transformation des MALT Lymphoms zu gDLBCL ist bisher nur wenig bekannt. Um den jetzigen Wissensstand über die molekulare Pathogenese der MALT Lymphome zu verbessern, haben wir während meiner Dissertation einen multidimensionalen Ansatz verfolgt, welcher diverse Aspekte dieser Krankheit beleuchtet hat.

Obwohl eine Vielzahl von Resultaten zeigen, dass Antigene eine entscheidenden Rolle bei der Entwicklung von MALT Lymphomen spielen, war bisher unklar, ob diese von oberflächenständigen Immunglobulinen der Tumorzellen erkannt werden können. Um die Antigen-Spezifität der Tumorimmunglobuline zu untersuchen, haben wir ELISA-Experimente zur systematischen Analyse des Antigenbindungsprofils durchgeführt. Hierzu haben wir humane wie auch murine Antikörper untersucht, welche von MALT Lymphomen stammen. Die Mehrzahl dieser Immunoglobuline besitzt ein breites, „polyreaktives“ Reaktionsspektrum, d.h. sie reagieren jeweils mit einer Vielzahl von Antigenen. Zusätzlich konnten wir zeigen, dass explantierte Tumorzellen über ihren polyreaktiven B-Zell Rezeptor verschiedene Antigene erkennen und durch sie zur Proliferation angeregt werden können.

Ein charakteristisches Merkmal der gastrischen MALT Lymphome ist, dass sie typischerweise durch eine grosse Anzahl von T-Helferzellen infiltriert werden, welche

Interleukin-4 und andere Th2 Zytokine produzieren. Demzufolge war es ein weiteres Ziel meiner Arbeit, die pathogene Rolle der tumorinfiltrierenden T-Zellen aufzuklären. Wir konnten zeigen, dass die Vermehrung der Tumorzellen durch die Gegenwart von eingewanderten $CD4^+$ T-Zellen in Abhängigkeit von der CD40/CD40L Interaktion verstärkt wird. Eine weitere entscheidende Erkenntnis war, dass ein signifikanter Teil der tumor-infiltrierenden $CD4^+$ T-Zellen aus funktionalen $CD25^+FoxP3^+$ regulatorischen T-Zellen (Treg) besteht. Diese Zellen wurden durch die Tumorzellen aktiv durch Sekretion der Chemokine CCL17 und CCL22 rekrutiert, welche Treg anlocken. Interessanterweise konnten wir beobachten, dass das Tumorwachstum *ex vivo* wie *in vivo* durch Depletion der $CD25^+$ T-Zellen oder der $CD4^+$ T-Zellen gehemmt werden konnte. Aus diesen Erkenntnissen schliessen wir, dass MALT Lymphome mindestens zwei Signale zur Proliferation benötigen. Eines dieser Signale wird durch die funktionalen polyreaktiven Immunoglobuline auf der Oberfläche übermittelt. Das andere Signal wird hingegen durch tumorinfiltrierende T-Zellen, insbesondere Treg, vermittelt, welche möglicherweise eine direkte Rolle bei der Stimulation des Tumorwachstums spielen.

In der MALT Lymphom-Forschung wurden bis heute überwiegend Expressionsveränderungen von proteinkodierenden Genen betrachtet, wobei neue Forschungsergebnisse zeigen, dass nicht-kodierende RNA, insbesondere microRNA (miRNA) in der Tumorentstehung eine entscheidende Rolle spielen können. Mit Hilfe eines microarraybasierten, genomweiten Ansatzes konnten wir miRNA-Expressionsmuster beschreiben, die mit der Entwicklung dieser Krankheit korrelieren. Ein Schwerpunkt lag dabei auf miR-203 und der tumorsuppressiven Funktion dieser miRNA bei der Progression von *Helicobacter*-abhängiger Gastritis zum MALT Lypmhom. Die Expression von miR-203 war im MALT Lymphomgewebe durch Hypermethylierung des miR-203 Locus signifikant herunterreguliert. Die experimentelle Überexpression von miR-203 in primären MALT Lymphomzellen unterdrückt das kürzlich identifizierte Target ABL1 und hemmt die Proliferation der Tumorzellen. Zudem konnten wir zeigen, dass die Inhibition der ABL1-Aktivität durch Imatinib die Vermehrung der MALT Lymphomzellen *ex vivo* hemmt und die Tumorentstehung im Tiermodell effizient unterdrückt. ABL1 scheint somit eine wichtige

Rolle bei der Entwicklung von MALT Lymphomen zu spielen; folglich kann Imatinib für die Behandlung von MALT Lymphompatienten in Betracht gezogen werden.

Unsere Genomanalyse hat weiterhin gezeigt, dass eine bestimmte Gruppe von MYC-reprimierten miRNAs in humanen gDLBCL im Vergleich zu MALT Lymphomen und Gastritis herunterreguliert sind. Wie wir in Gewebearray-Analysen immunhistochemisch zeigen konnten, korreliert eine aberrante MYC-Expression tatsächlich mit der hochgradigen Transformation des MALT Lymphoms. In der Tat kann die Proliferation von DLBCL Zellen durch experimentelle Re-Expression der MYC-regulierten miRNAs deutlich reduziert werden. Die miR-34a hat dabei die stärksten tumorsuppressiven Eigenschaften in DLCBL Zelllinien gezeigt, welche wir auf eine Dysregulierung des miR-34a Targets FOXP1 zurück-führen konnten. Übereinstimmend mit diesen Resultaten haben wir eine Korrelation zwischen FOXP1 Überexpression und hochgradiger Transformation des MALT Lymphoms beobachtet; zudem hat ein transienter Knockdown von FOXP1 in DLBCL Zelllinien die Proliferation von Tumorzellen deutlich gehemmt. Unsere Ergebnisse stellen somit einen Zusammenhang her zwischen der Überexpression von MYC, der daraus resultierenden Repression von miR-34a und anderen MYC-reprimierten miRNAs, und der Überexpression von FOXP1, welche mit der hochgradigen Transformation des MALT Lymphoms korrelieren und diese möglicherweise verursachen können.

1 INTRODUCTION

1.1 The biology of gastric MALT lymphoma

In 1983, Isaacson and Wright first described a novel form of extranodal non-Hodgkin's B-cell lymphoma arising in mucosal organs with a distinct indolent clinical course.[1] The term 'MALT lymphoma' was established owing to their observation that the histological features recapitulated those of the Peyer's patch, a component of mucosa associated lymphoid tissue (MALT) found in the small intestines. It was not until 1994 that MALT lymphoma was officially recognised as a discrete entity in the group of marginal zone B-cell lymphomas by the Revised European-American Lymphoma (REAL) classification. In the current world health organisation (WHO) classification system, MALT lymphomas are grouped with splenic and nodal marginal-zone lymphomas based on the normal cell counterpart of these B-cell neoplasms (the marginal zone B-cell). Despite this common histological feature, MALT lymphoma differs from its nodal and splenic counterparts with respect to pathological and clinical behaviour.

MALT lymphomas arise in the context of pre-existing prolonged lymphoid proliferation in a wide variety of mucosal sites that are normally devoid of organised lymphoid tissue. The stomach is the most commonly affected organ (70% of cases), while other tumour sites include the lung (14%), ocular adnexa (12%), thyroid (4%) and small intestine (1%). The lymphoid tissue, from which the neoplastic B-cell clones emerge, is acquired in response to chronic infection or autoimmune disease and therefore exemplifies the close pathogenic link between chronic inflammation and tumour development. In the case of gastric lymphoma, persistent infection with the bacterium *Helicobacter pylori* is recognised as the etiological agent responsible for creating the chronic local inflammatory environment necessary for this type of lymphoma to develop.

MALT lymphomas comprise 7-8 % of all B-cell lymphomas making them the third most common subtype, after diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma.[2] Gastric MALT lymphoma represents the most frequent lymphoma of the gastrointestinal

tract accounting for least 50% of all gastric haematopoietic malignancies and up to 5% of all gastric neoplasms.[3, 4] In the case of gastric MALT lymphoma, the disease is remarkably indolent and tends to remain localised to the stomach for long periods. The ten-year survival rate for gastric MALT lymphoma is close to 90% with a disease-free survival of approximately 70%.[5, 6] However, MALT lymphomas can progress and transform into aggressive high grade tumours (gDLBCL), whereby the ten-year survival rate falls to approximately 42%.[5]

1.1.1 Clinical and histopathological features

The neoplastic B cells of MALT lymphoma share the cytological features and immunophenotype ($CD20^+$, $CD21^+$, $CD35^+$, IgM^+, IgD^-) of marginal zone B cells.[7] The tumour cells can resemble follicle-centre centrocytes (centrocyte-like cells) or small lymphocytes (monocytoid B cells) (Figure 1.1d, e). Scattered large (transformed) lymphoma cells (immunoblasts and centroblast-like cells) are also common. The infiltrate occurs in the marginal zone of reactive B-cell follicles and extends into the interfollicular region.[8]

A characteristic feature of MALT lymphoma is the presence of lymphoepithelial lesions (LELs), defined by the invasion and destruction of individual mucosal glands or other epithelial structures by clusters of (usually three or more) neoplastic cells (Figure 1.1a, c). Plasma cell differentiation occurs in a third of all MALT lymphoma cases and tends to be maximal beneath the surface epithelium. Follicular colonisation is another phenomenon of MALT lymphoma and describes the extension of tumour cells into germinal centres (GCs) of non-malignant reactive B-cell follicles (Figure 1.1b).[9] Several studies have reported that gastric MALT lymphomas are infiltrated by large numbers of T-helper cells expressing interleukin-4 (IL4) and other T-helper cell type 2 (Th2) cytokines (see section 1.1.4.2).[10-12] Histologic transformation of MALT lymphoma to gDLBCL is characterised by increased numbers of large blast cells that form confluent aggregates or sheets that can ultimately efface any residual preceding low grade tumour.[13]

Diagnosis of gastric MALT lymphoma is made by morphologic analysis of endoscopic mucosal biopsy samples. Histologic grading of gastric biopsies is usually

performed according to a scoring system developed by Wotherspoon in 1993 (Table 1.1).[14] For cases in which the diagnosis remains ambiguous, immunophenotyping or molecular genetic analysis to assess B-cell clonality may be employed. The later is accomplished by polymerase chain reaction (PCR)-based amplification of rearranged VDJ sequences of the heavy-chain immunoglobulin (*Ig*) gene. In terms of immunophenotype, there is no specific marker for MALT lymphoma at present. Tumour cells typically express IgM, CD20, CD79a, CD21, CD35 and show light chain restriction. MALT lymphoma can be differentiated from other B-cell lymphomas in the stomach, namely mantle cell lymphoma, lymphocytic lymphoma and follicular lymphoma, by the absence of characteristic markers CD5, CD10, CD23 and cyclin D1.

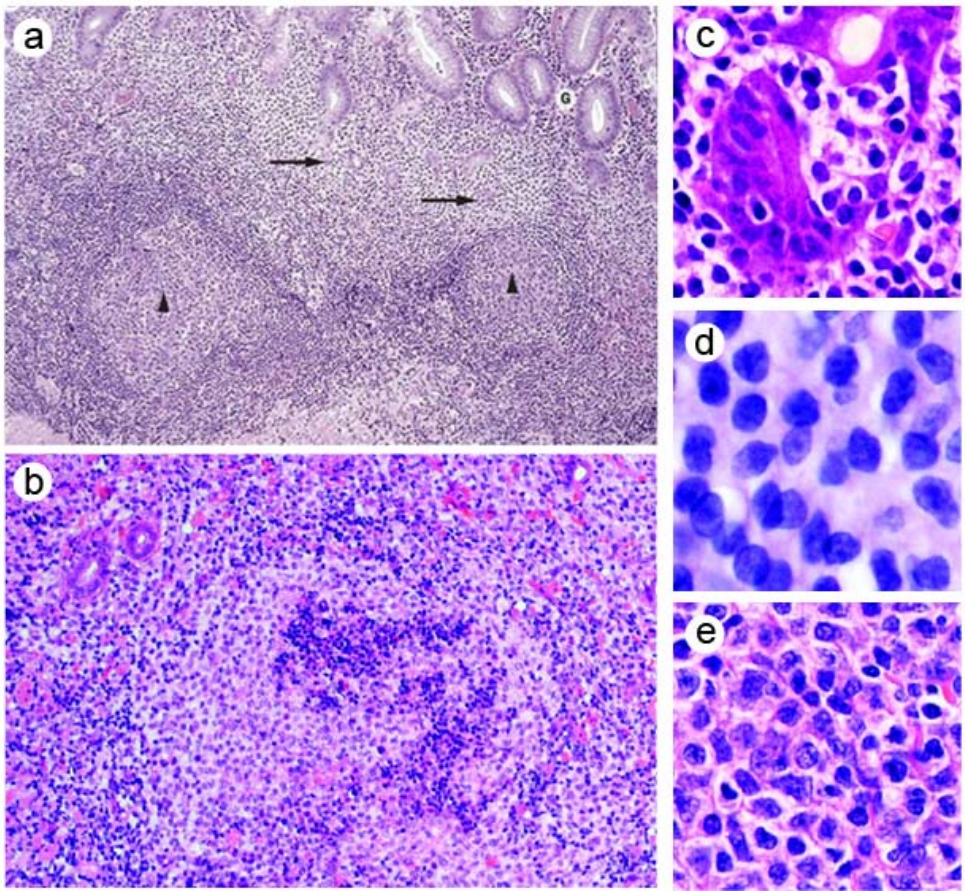

Figure 1.1 Morphological features of gastric MALT lymphoma. **a** Centrocyte-like neoplastic B cells surround reactive B-cell follicles (arrowheads) and invade the gastric glandular epithelium (G), resulting in lymphoepithelial lesions (LELs; arrows).[15] **b** Follicular colonisation leads to replacement of the B-cell follicle by neoplastic B cells with moncytoid appearance due to abundant pale staining cytoplasm.[14] A rim of normal mantle zone is retained.[16] **c** Small neoplastic B-cells with a narrow rim of clear cytoplasm forming LELs.[16] **d** Lymphoma cells resembling small lymphocytes with condensed chromatin.[16] **e** Tumour cells with nuclei resembling those of centrocytes, but with more abundant cytoplasm.[16]

Although most gastric MALT lymphomas present as localised disease at diagnosis, the tumour may disseminate both locally, to form multifocal lesions, and systemically.[17] A prospective study involving over 200 patients detected involvement of the regional lymph nodes in 32% of cases and secondary tumours in extranodal sites in 11% cases.[18] Dissemination to the regional lymph nodes and the spleen typically involves the marginal zone around B-cell follicles.[19] The tendency of dissemination to other parts of the gastrointestinal tract or the splenic marginal zone is due to high expression of the mucosal homing receptor $\alpha_4\beta_7$ integrin by the lymphoma B cells.[20, 21]

Table 1.1 Histological scoring for diagnosis of MALT lymphoma. Adapted from[14].

Wotherspoon index	Description	Histologic features
0	Normal mucosal membrane	Scattered plamsa cells in lamina propria, no lymphoid follicles
1	Chronic active gastritis	Small clusters of lymphocytes in lamina propria, no lymphoid follicles, no lymphoepithelial lesions (LELs)
2	Chronic active gastritis with lymphoid follicle formation	Prominent lymphoid follicles with surrounding mantle zone and plasma cells, no LELs
3	Suspicious lymphoid infiltrate in lamina propria, probably reactive	Lymphoid follicles surrounded by small lymphocytes that infiltrate diffusely in lamina propria and occasionally into epithelium
4	Suspicious lymphoid infiltrate in lamina propria, probably lymphoma	Lymphoid follicles surrounded by centrocyte-like cells that infiltrate diffusely in lamina propria and into epithelium in small groups
5	MALT lymphoma	Presence of dense diffuse infiltrate of centrocyte-like cells in lamina propria with prominent LELs

1.1.2 The *Helicobacter pylori* connection

The paradox of MALT lymphoma arising in an organ naturally devoid of organised lymphoid tissue, was first resolved by Wotherspoon et al.[22] in 1991, in a study linking *H. pylori* infection with gastric MALT lymphoma. *H. pylori* is a gram-negative microaerophilic bacterium that colonises the human gastric epithelium of approximately half the world's population.[23] Multiple lines of evidence implicate a direct role for *H. pylori* in the pathogenesis of MALT lymphoma. First, *H. pylori* is present in the gastric mucosa of most cases of gastric MALT lymphoma, with infection rates ranging from 60% to over 90% of

cases.[22, 24-26] Second, gastric MALT lymphoma has the highest incidence in geographical regions with high prevalence of *H. pylori* infection.[3] Third, experimental infection of mice with various *Helicobacter* strains induces gastric MALT lymphoma-like lesions.[27] Finally, a large case-control study provided definitive evidence for an association between previous *H. pylori* infection and the development of gastric, but not non-gastric lymphoma.[28] Interestingly, the frequency of *H. pylori* positivity at diagnosis is lower in gastric lymphoma than in chronic gastritis or peptic ulcer indicating loss of the ecological niche of the bacterium upon disease progression.[24]

The notion of *H. pylori* as a gastric carcinogen is reinforced by the clinical and histological remission of some cases (approximately 75%) of low grade MALT lymphomas after *H. pylori* eradication by antibiotic therapy. [14, 29-31] To date, gastric MALT lymphoma is the only malignancy for which antibiotics are used as the first-line treatment. Direct evidence implicating *H. pylori* as the driving force in MALT lymphomagenesis came from *in vitro* studies wherein, crude tumour cell suspensions proliferated upon addition of heat-killed, whole-cell preparations of *H. pylori*.[32] Importantly, this response was demonstrated to be specific for only one bacterial strain per case of lymphoma.[32] In contrast, control experiments using nodal lymphoma cells failed to respond to any strain of *Helicobacter*.

While *H. pylori* is accepted as the dominant human gastric bacterial pathogen in MALT lymphoma, a small percentage of human infections have been linked to another *Helicobacter* species, referred to as *H. heilmannii*. While this organism is the primary strain seen in primates, pigs and carnivorous mammals, only a small percentage of infections in humans (0.5-6%) have been attributed to *H. heilmanni* and transmission from animals has been suggested.[33-35] Despite this low prevalence, an association with gastric MALT lymphoma has been demonstrated and eradication of the organism leads to tumour regression identical to that of *H. pylori*.[36]

Although the causal relationship between *H. pylori* infection and gastric MALT lymphoma is well established, the reason why only a minority of infected individuals (<1%) develop MALT lymphoma is less clear. At present, no host susceptibility factor or

environmental factor predisposing a patient to MALT lymphoma has been revealed to shed light on the enigma. A recent study investigated, for the first time, the potential role of the host's genetic background as a risk factor for gastric MALT lymphoma.[37] Germline variations of *MALT1* (which encodes MALT lymphoma translocation protein 1) were examined in a large cohort of patients with gastric MALT lymphoma but failed to demonstrate a clear link to disease susceptibility.[37]

In addition to potential host and environmental factors, there are specific bacterial virulence genes that may determine the disease outcome. A close link exists between gastric carcinoma and *Helicobacter* strains that harbour the *cag*-pathogenicity island (*cag* PAI) and its secreted effector molecule CagA, as well as several other virulence factors such as IceA and the vacuolising cytotoxin VacA. In contrast, characteristics of *H. pylori* strains associated with MALT lymphoma are less clear. Existing studies have focused mainly on the role of CagA and its prevalence in strains isolated from MALT lymphoma. Epidemiological data from one study indicated that *cagA*-positive *H. pylori* is present in the gastric mucosa of most, if not all, patients with MALT lymphoma.[38] In contrast, another group reported no significant association of *cagA*-positive *H. pylori* strains with lymphoma development.[39] Subsequent studies have compared the *cagA* genotype between low and high grade MALT lymphoma and found that indeed the incidence of *cagA*-positive *H. pylori* infection in gDLBCL was significantly higher than in low grade MALT lymphoma or gastritis.[40, 41] Altogether, these observations allude to an important role for *cagA*-positive *H. pylori* in the development and/or progression of gastric MALT lymphoma.

1.1.3 Treatment and follow-up

1.1.3.1 Treatment of low grade gastric MALT lymphoma

Triple therapy, consisting of a combination of antibiotics, (clarithomycin, amoxicillin, tetracycline and/or metronidazole) with a proton pump inhibitor has become the first-line option for patients with localised, *H. pylori*-positive MALT lymphoma. *Helicobacter* eradication therapies have revolutionised the natural course of MALT lymphoma. In the past, surgical excision either alone or followed by radiotherapy or postoperative chemotherapy, was considered the gold-standard treatment until the early

1990s. These approaches resulted in five-year survival rates as high as 90%.[5] The multifocal nature of this disease often necessitated a total gastrectomy (rather than partial resection) which carries a risk of morbidity and the potential to severely impair the patient's quality of life. Surgical intervention for gastric MALT lymphoma is now only reserved for patients with local complications, such as GI haemorrhage.

Since the introduction of *H. pylori* eradication therapy in 1993,[14] several independent studies have confirmed its efficacy in the treatment of localised low grade MALT lymphomas.[30, 42, 43] Tumours confined to the mucosa and submucosa are treated most effectively; whereas, tumours with involvement of the stomach wall or perigastric lymph nodes or lymphoma cases positive for the t(11;18)(q21;q21) translocation are typically refractory to eradication therapy.[44-46] There is no standard treatment for patients not responding to eradication therapy; those with a negative *H. pylori* status are usually referred for radiation, chemotherapy, or immunotherapy, or combinations of these. Relatively low doses of involved-field radiotherapy (30-40 Gy over 4 weeks) can be applied to the stomach with good results.[47] Immunotherapy with anti-CD20 monoclonal antibodies and/or chemotherapy is usually set aside only for patients with disseminated disease.[48]

Several studies have provided molecular evidence that monoclonal tumour cells can persist long after complete histological remission following successful *H. pylori* eradication.[29, 30, 49] Such patients are considered to have 'minimal residual disease' and are at a significantly higher risk of relapse.[50] However, a conservative 'watch and wait' strategy with regular endoscopies and biopsies is the recommended approach for patients with minimal residual disease.[51] In general, relapse rates in patients treated for the infection alone vary between 1-20%.[42, 52-54] Serial gastric biopsies are therefore mandatory for the surveillance of patients following eradication therapy alone. Endoscopic examination is recommended 2-3 months after termination of antibiotic treatment to confirm *H. pylori* eradication, followed by endoscopy every 6 months for 2 years.[55]

1.1.3.2 Treatment of gastric diffuse large B-cell lymphoma

Nearly 60% of gastric lymphomas are high grade lesions with or without a low grade MALT component.[56] These lymphomas are generally *H. pylori* independent and are therefore resistant to antibiotic therapy. However, like MALT lymphoma, treatment of gDLBCL has undergone a dramatic shift away from surgical management with the current treatment options favouring chemotherapy and radiation therapy according to the extent of disease. The most common regimen for localised disease is immuno-chemotherapy entailing a short course of R-CHOP (Rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone) for three or four cycles followed by involved-field radiotherapy. Evidence has recently surfaced that some cases of high grade gastric MALT lymphoma might still be responsive to *H. pylori* antigenic drive and may be cured with eradication therapy.[29, 57] However, prospective studies with large patient numbers are warranted to validate these results and may help to identify factors that predict a favourable response to *H. pylori* eradication in order to avoid more aggressive treatment strategies.

1.1.4 Pathogenesis of gastric MALT lymphoma

The evolution of gastric MALT lymphoma is a multistep process that results in transformation from a reactive polyclonal B-cell population to a neoplastic monoclonal lymphoproliferation (Figure 1.2). *H. pylori* infection induces chronic activation of the immune system and triggers sustained lymphoid proliferation. Gastric MALT lymphoma therefore constitutes a unique type of infection-associated lymphoma, in which the infectious agent does not directly infect and transform lymphoid cells. In this context rather, the microbial pathogen is considered a chronic source of antigen. The molecular mechanisms underlying this indirect lymphomagenesis await precise elucidation. Mounting evidence implicates factors such as, signalling via surface Ig, T-cell derived costimulatory signals and the acquisition of genetic abnormalities as important driving forces in the early stages of MALT lymphoma pathogenesis.

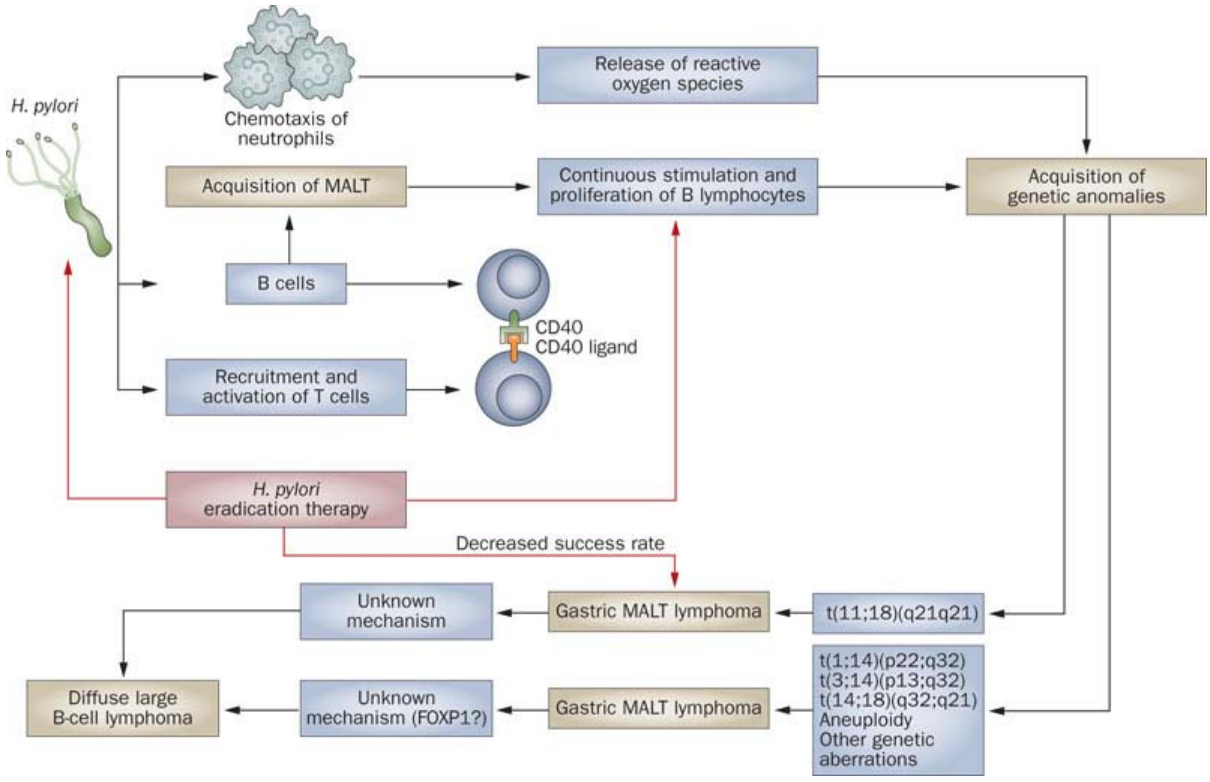

Figure 1.2 Hypothetical model depicting the multistage development of gastric MALT lymphoma. Persistent *H. pylori* infection attracts B cells, T cells and neutrophils to the gastric mucosa. B-cell proliferation is driven by CD40–CD40 ligand interaction with H. pylori-activated, reactive T cells, as well as by cytokines. The chronic proliferative state of these B cells, as well as neutrophil-mediated release of reactive oxygen species in areas of chronic inflammation, induces additional oncogenic events that eventually make lymphoproliferation independent of antigenic stimulation. *H. pylori* eradication therapy inhibits (red arrows) these tumour-promoting processes, although t(11;18)(q21;q21)-positive MALT lymphomas are less likely to respond to this therapy than are tumours without this translocation. Additional genetic alterations in t(11;18)(q21;q21)-negative MALT lymphomas can ultimately result in transformation to clinically aggressive diffuse large B-cell lymphoma. Reproduced from Sagaert et al., 2010.[15]

1.1.4.1 Gastric MALT acquisition

The acquisition of organised lymphoid tissue is the first step towards lymphoma development in the stomach. Under normal physiological conditions, the gastric mucosa is devoid of organised lymphoid tissue. *H. pylori*, unlike other microorganisms, has evolved the unique ability to survive in the stomach by secreting the enzyme urease that converts urea to ammonia leading to reduced acidity of the stomach. With age, the combination of infection and decreased acidity of the stomach elicits the accumulation of lymphocytes and thus the formation of MALT.

1.1.4.2 Role of Infiltrating T cells

Current evidence indicates that MALT lymphoma cells do not grow autonomously, rather they are dependent on T-helper cell-derived activation and proliferation signals. MALT lymphomas are invariably infiltrated by high numbers of activated, immunocompetent memory T cells, with an estimated ratio of $CD4^+/CD8^+$ cells of approximately 4:1 (Figure 1.3).[10, 11] Koulis et al.[58] demonstrated that the proliferating fraction of tumour-infiltrating cells were exclusively $CD4^+$ helper T cells. Antigen-specific activation of infiltrating T cells was demonstrated by their expression of CD40-ligand,[10, 58] a co-stimulatory molecule necessary for effective B-cell/T-cell interaction. Tumour-infiltrating T cells highly express the immunocompetence marker CD28, which binds to the B7-related surface proteins CD80 and CD86 on antigen-presenting cells and permits optimal activation of T cells upon antigen recognition.[11, 58] Expression of the costimulatory molecules CD80 and CD86 has been demonstrated in MALT lymphomas cells.[58-60]

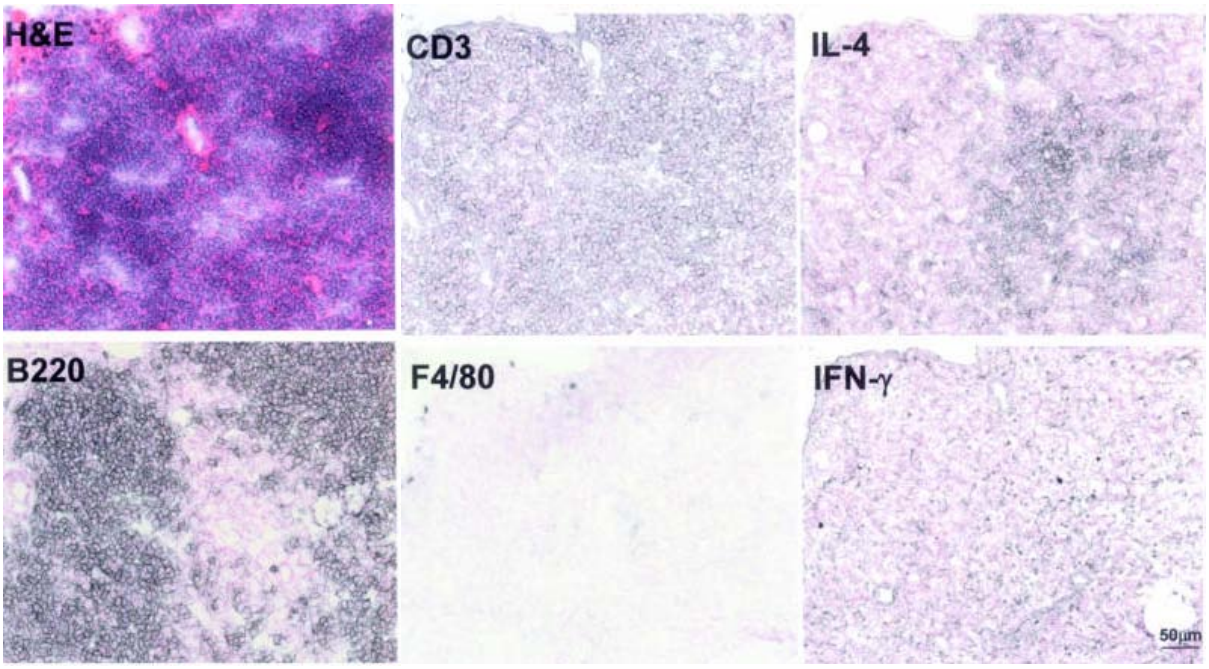

Figure 1.3 Tumour-infiltrating T cells are predominantly $CD4^+$ and of the type 2 helper T-cell (Th2) Type. Serial cryosections of a representative murine lymphoid aggregate were stained blue-grey with monoclonal antibodies against the indicated antigens and counterstained with nuclear fast red. $B220^+$ B cells constitute the predominant cell type. $CD3^+$ T cells are abundant, whereas $F4/80^+$ macrophages are present at very low numbers and found only around the edges of the tumour. Lymphoid aggregates are colonised by $CD3^+$ T cells, but not $F4/80^+$ macrophages. Tumour-infiltrating T cells are mostly $CD4^+$ and produce IL4, but not IFN-γ. Reproduced from Mueller et al., 2005.[11]

Several lines of evidence suggest that *H. pylori*-specific activated tumour-infiltrating T cells provide growth signals to the tumour B cells. Hussell et al.[32, 61] first demonstrated that cultures of explanted, unsorted tumour cells proliferate in response to *H. pylori* extract. Removal of tumour-associated T cells from the culture system abrogated proliferation, suggesting that their growth is driven by the presence of T cells.[61] This reaction has been shown to be strain specific and site dependent indicating that T cells are responding to *H. pylori*-derived antigens.[62] In contrast, high grade MALT lymphomas do not respond to *H. pylori* stimulation *in vitro*.[62] These observations partly explain the proclivity of low grade tumours to remain localised to the primary site and regress after *H. pylori* is eradicated with antibiotics.

Greiner et al.[63] showed that the tumour cells are responsive to CD40 stimulation (via ligation of the CD40 receptor by an agonistic antibody) in the presence of Th2 derived cytokines IL4 and IL10, but not Th1 cytokines IFN-γ or IL2 (Figure 1.4). Combinations of both IL4 and IL10 in this CD40 culture system synergised to induce marked proliferation of low grade MALT lymphoma cells.[63] In contrast, high grade MALT lymphomas responded significantly to CD40 signal alone and in combination with either Th1 or Th2 derived cytokines.[63] In line with this evidence, infiltrating T cells of low grade MALT lymphoma typically express high levels of CD40L and Th2 cytokines (Figure 1.3), with the neoplastic B cells being positive for CD40.[10, 11, 58] Most high grade tumours have been shown to lack CD40L expression which again implies a different role for infiltrating T cells in the high grade scenario.[59] Further supporting the critical role of a Th2 helper response in MALT lymphomagenesis, is the fact that these lymphomas only develop in mouse strains with a genetic predisposition towards Th2 polarisation, such as BALB/c.

D'Elios et al.[64] observed that T cells derived from MALT lymphoma exhibited both defective perforin-mediated cytotoxicity and poor ability to induce apoptosis. Deficient cytotoxic control of B-cell growth thus provides a plausible explanation for their enhanced helper activity on B-cell proliferation. Current evidence therefore sustains the notion that tumour-infiltrating T cells do not represent a host immune response, but rather are part of a T-cell dependence underlying the antigen-driven development of gastric MALT lymphomas.

Additional studies are clearly necessary to further characterise this infiltrating T-cell population and elucidate the relative contribution of T-helper derived signals to MALT lymphoma growth *in vitro* and *in vivo*.

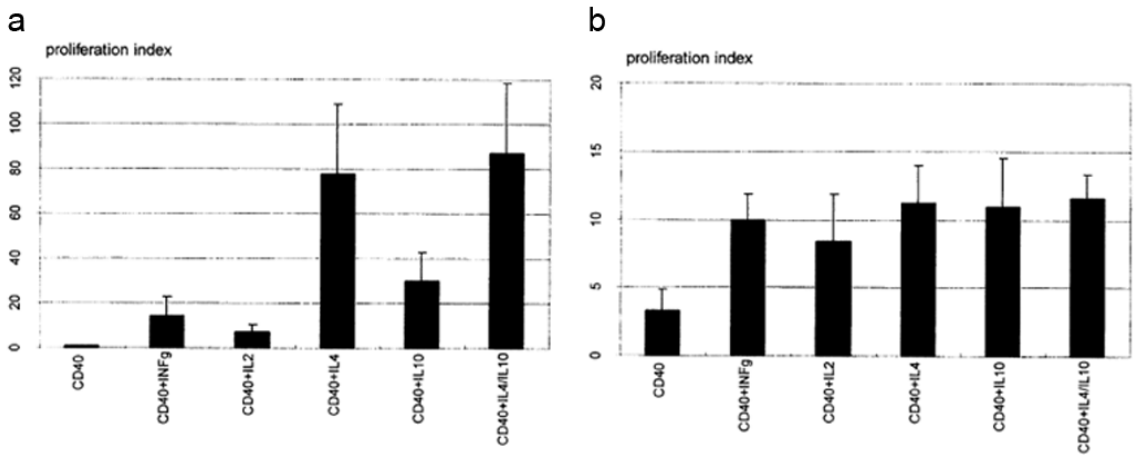

Figure 1.4 Induction of low grade (a) and high grade (b) MALT lymphoma B-cell proliferation by anti-CD40 and cytokines. 5 x 10^4 tumour B cells from 6 low grade and 5 high grade MALT lymphoma patients were cultured in triplicate with or without cytokines and anti-CD40 as indicated. B-cell proliferation was determined at day 5 of culture by the addition of [^{3}H] thymidine during the last 16 hours of the culture period. The proliferation index is expressed as cpm assay/cpm medium. The results give the cumulative mean value from three independent proliferation tests performed for each patient. Each error bar gives the standard deviation of the respective cumulative mean. Reproduced from Greiner et al., 1997.[63]

1.1.4.3 MALT lymphoma as an antigen-driven neoplasm

Direct and indirect evidence alludes to a pathogenic role for antigen in MALT lymphomagenesis, whereby tumour cells may receive signals via antigenic stimulation through their B-cell receptor (BCR). Direct evidence comes from the observation that a proportion of low grade gastric MALT lymphomas respond favourably to bacterial eradication alone.[14, 65] The concept of an antigen-driven lymphomagenesis is indirectly supported by analysis of the variable part of the Ig heavy chain (V_H) gene from tumour cells. MALT lymphoma B cells carry rearranged and somatically mutated *Ig* genes, confirming their origin from antigen activated memory B cells.[66, 69] Multiple studies examining antigenic selection of the V_H genes have reported evidence of positive selective pressure, suggesting that MALT lymphoma B cells have undergone the germinal centre reaction and affinity maturation.[63, 67, 68] Preferential use of V_H family genes (V_H4, V_H3 and V_H1) previously associated with autoreactivity and polyreactivity has also been noted.[66, 69] In addition,

ongoing mutation, as indicated by intraclonal variation of the Ig sequence, exists in low grade tumours which suggests continued antigenic stimulation is important in clonal B-cell expansion.[68, 70, 71]

The occurrence of somatic hypermutation and intraclonal variation in MALT lymphoma strongly bolsters a role for antigen, given these processes are antigen-driven. Of note, the ongoing mutation rate dissipates as low grade tumours progress, and eventually disappears in some gDLBCLs.[68, 72] The lack of ongoing mutation in high grade tumour cells may reflect the loss of ability to respond to direct antigen stimulation. This is currently thought to be the result of an accumulation of genetic defects, rendering the lymphoma less dependent on antigenic stimulation over time. A confounding feature of MALT lymphoma is that the *Ig* gene locus undergoes somatic hypermutation yet avoids class switch recombination (CSR), indicating that IgM^+ tumour cells are favoured by selective forces. Retention of IgM expression in MALT lymphoma is thought to be due to their propensity to undergo an aberrant form of switch recombination, i.e. only one rather than two switch regions are involved, leading to an abnormal rearrangement of the *IgH* switch region and conservation of IgM status.[73]

Despite strong support for antigen as a pathogenic factor in MALT lymphomagenesis, the search for a target ligand has proven to be difficult and has yielded controversial results. This may be attributable to the different approaches used to generate tumour-derived antibody and the subsequent screening methods applied. The tumour Ig specificity may also depend on the anatomical location from which the lymphoma originates. Hussel et al.[172] first addressed the issue of Ig specificity using anti-idiotypic antibodies derived from three cases of gastric MALT lymphoma; the authors detected polyreactivity in one case and described reactivity towards follicular dendritic cells and mucosal post capillary venules in the others. Greiner et al.[75] also did not identify a specific antigen, but concluded that the target ligand is a common antigen of IgA^+ and IgM^+ mucosal B cells. Lenze et al.[76] screened seven single-chain fragment variable antibodies derived from gastric and non-gastric MALT lymphomas but failed to identify binding partners for the majority of the Igs analysed. Finally, Bende et al.[66] reported that many MALT lymphomas express B-cell receptors with strong homology to

rheumatoid factors (RFs), i.e. antibodies with auto-IgG binding capacity. Specifically, the authors found that 8 of 45 gastric (18%), 13 of 32 salivary gland (41%) and none of the 19 (0%) pulmonary MALT lymphomas expressed antigen receptors with strong RF homology; experimentally they confirmed this finding for 7 of 10 antibodies analysed.[66] However, the bulk of these antibodies were derived from parotid gland lymphomas, with only 2 being derived from gastric tumour Igs.[66] A more comprehensive study is therefore desired to assess the antigen specificity of gastric MALT lymphoma Ig and to further determine whether signalling through the B-cell receptor directly contributes to the proliferation of these malignant lymphocytes.

1.1.4.4 Genetic abnormalities

H. pylori infection triggers not only an immunological response that stimulates the growth of neoplastic B cells, but also elicits an inflammatory response by attracting and activating neutrophils. The subsequent release of reactive oxygen species (ROS) by neutrophils are suspected to cause a wide range of genetic damage, principally double-strand DNA breaks.[77] Moreover, the inherent genetic instability of B cells during somatic hypermutation and class-switch recombination further exacerbates the risk of oncogenic DNA damage[78] during the prolonged proliferation of B cells induced by chronic inflammation.

A characteristic spectrum of genetic defects in MALT lymphomas have been detected in recent years. Abnormalities include trisomy of chromosomes 3, 7, 12 and 18 as well as the chromosomal translocations t(1;14)(p22;q32), t(14;18) (q32;q21), t(11;18)(q21;q21) and t(3;14)(p13;q32) which result in *IGH-BCL10*, *IGH-MALT1*, *BIRC3-MALT1* and *IGH-FOXP1* rearrangements, respectively. These genetic aberrations occur at variable frequencies according to the anatomic location of the tumour (Table 1.2). It is noteworthy that the shared outcome of the majority of these genetic aberrations is the constitutive activation of the nuclear factor-κB (NF-κB) signalling pathway (Figure 1.5). NF-κB is a key transcription factor in immunity, as it regulates the expression of a number of genes involved in B-cell proliferation and survival.[79, 80] Constitutive activation of NF-κB by

genetic abnormalities results in uncontrolled B-cell proliferation and thus contributes to the neoplastic transformation of the B-cell clone.

Table 1.2 Frequency of chromosomal translocations in MALT lymphoma. Adapted from[8, 15].

Translocation	Fusion transcript	Mechanism of upregulated NF-κB activation	Frequency in MALT lymphoma
t(11;18)(q21;q21)	*BIRC3-MALT1*	Polyubiquitinylation of NEMO	Stomach 6-26%; Lung 31-53%; Ocular adnexa 0-10%; Salivary glands 0-5%; Thyroid 0-17%
t(14;18)(q32;q21)	*IGH-MALT1*	MALT1 oligomerisation (Bcl-10 dependent)	Stomach 1-5%; Lung 6-10%; Ocular adnexa 0-25%; Salivary glands 0-16%; Thyroid 0%
t(1;14)(p22;q32)	*BCL10-IGH*	MALT1 oligomerisation	Stomach 0%; Lung 2-7%; Ocular adnexa 0%; Salivary glands 0-2%; Thyroid 0%
t(3;14)(p13;q32)	*FOXP1-IGH*	Unknown	Stomach 0%; Lung 0%; Ocular adnexa 0-20%; Salivary glands 0%; Thyroid 0-50%

Translocations

The translocation t(11;18)(q21;q21) represents the most frequent structural chromosomal abnormality in gastric MALT lymphoma, accounting for up to 26% of cases.[81] In contrast to t(11;18)-positive tumours, t(14;18), t(1;14) and t(3;14) translocations mainly arise in non-gastric tissues and are therefore not discussed further. Remarkably, in most t(11;18)-positive cases it appears to be the sole chromosome aberration.[82, 83] In contrast, cases lacking this translocation exhibit a wide range of chromosomal aberrations.[83, 84] Lymphomas with this translocation are invariably refractory to *H. pylori* eradication therapy and are more likely to show dissemination to regional lymph nodes or distal sites.[85, 86] Initially, it was believed that t(11;18)-positive tumours rarely, if ever, evolved into gDLBCL.[85] However, recent work has shown that gDLBCLs exhibit this translocation at equivalent frequencies to low grade gastric MALT lymphomas and therefore its presence does not exclude high grade transformation.[87] The translocation t(11;18)(q21;q21), fuses the amino-terminus of the *BIRC3* (baculovirus inhibitor of apoptosis protein repeat-containing protein 3) gene (on chromosome 11q21) to the carboxyl-terminus of the *MALT1* gene (on chromosome 18q21) to create the chimeric BIRC3-MALT1 protein. BIRC3 suppresses apoptosis by inhibiting the activity of caspases 3, 7 and 9, whereas MALT1 is involved in antigen-receptor-mediated NF-κB activation. How the BIRC3-MALT1 fusion precisely contributes to MALT lymphoma development remains to be eludicated.

Trisomy 3

Cytogenetic and fluorescence *in situ* hybridisation (FISH) studies have revealed a high frequency of trisomy 3 in gastric MALT lymphoma.[88, 89] However, it is also the most common form of aneuploidy described in other types of non-Hodgkin's lymphoma. The genetic mechanism by which trisomy 3 may contribute to lymphomagenesis has not yet been experimentally addressed. However, an increased gene dosage effect resulting from higher copy numbers of genes relevant to lymphoma development is likely to explain the biological consequences underlying chromosomal trisomies.[90] Several promising candidate genes are located on chromosome 3 which have been implicated in lymphomagensis, such as the proto-oncogene *BCL6* and the transcription factor *FOXP1* (described in more detail in section 1.1.5).

Epigenetics

In recent years, evidence for the involvement of epigenetic mechanisms in the initiation and development of MALT lymphoma has began to emerge. Attention has focused solely on the epigenetic phenomenon of DNA hypermethylation which entails the enzymatic addition of a methyl group to the carbon 5 position of a cytosine that precedes a guanosine in the DNA sequence (termed CpG dinucleotide). This covalent modification of DNA is catalysed by DNA methyltransferases (DNMTs) and is associated with transcriptional silencing.[91] Min et al.[92] demonstrated CpG hypermethylation of the tumour suppressor genes *TP16* and *TP57* in 41.7% and 29.2% of low grade MALT lymphoma cases, respectively. A different group analysed the methylation status of eight CpG islands, including those of *TP15*, *TP16*, *TP73*, *hMLH1*, *DAPK*, *MINT1*, *MINT2* and *MINT31* in *H. pylori* dependent and independent MALT lymphomas[93] The authors found that *H. pylori* dependent MALT lymphomas exhibited at least four methylated genes, while *H. pylori* independent cases displayed lower methylation levels with less than two methylated genes.[93] This suggests that chronic inflammatory conditions induced by *H. pylori* infection may promote DNA methylation in reactive lymphocytes. Other epigenetic mechanisms such as covalent modification of histones or microRNA mediated gene regulation have not yet been studied in the context of MALT lymphoma.

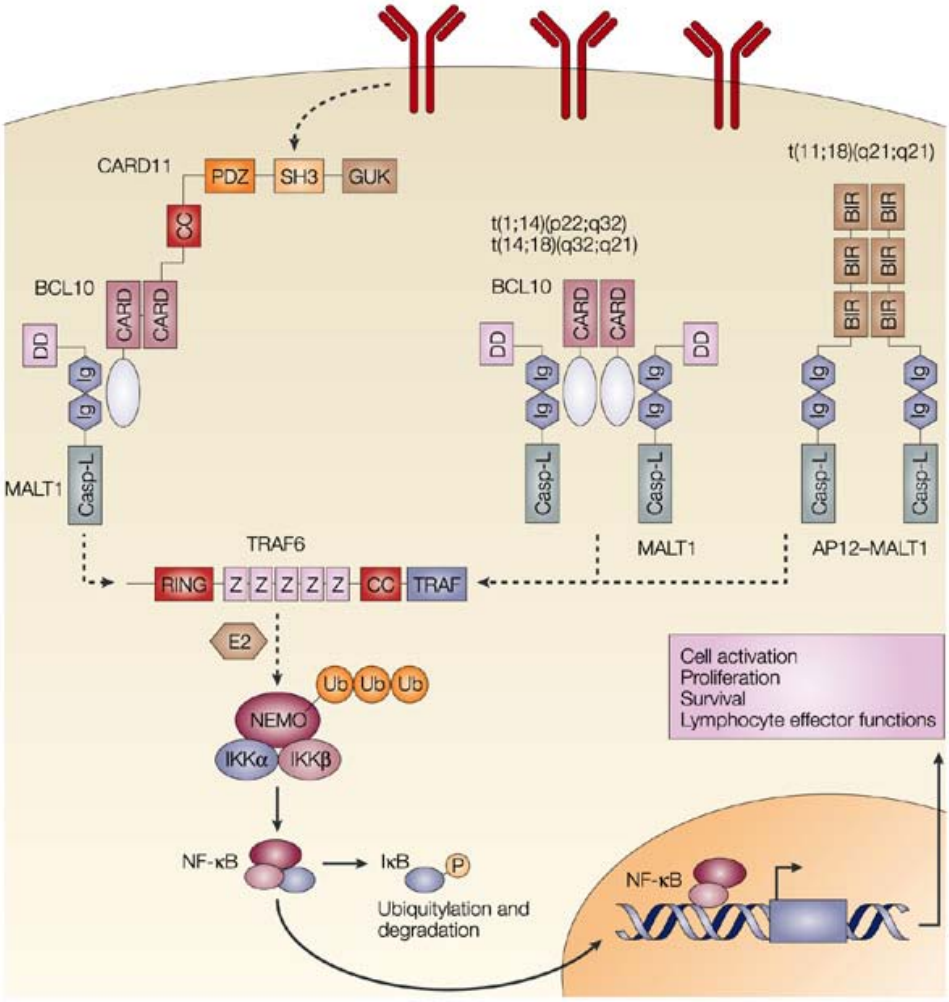

Figure 1.5 The different chromosomal translocations involved in MALT lymphoma affect a common molecular pathway. Following antigen-receptor stimulation, CARD11 is activated to recruit BCL10 through caspase recruitment domain (CARD)–CARD interactions, and this induces BCL10 oligomerisation. BCL10 then binds the immunoglobulin (Ig)-like domain of MALT1 through a short region downstream of its CARD and induces MALT1 oligomerisation. Oligomerised MALT1 binds to and activates tumour-necrosis factor receptor associated factor 6 (TRAF6). Activated TRAF6 interacts with a ubiquitin-conjugating enzyme (E2) and mediates polyubiquitylation of nuclear factor-κB (NF-κB) essential modulator (NEMO). Multi-ubiquitylated NEMO induces the activation of IKKα and IKKβ, which causes phosphorylation and degradation of IκB and the release of NF-κB. NF-κB then translocates to the nucleus and transactivates genes important for lymphomagenesis. In MALT lymphomas involving t(1;14)(p22;q32), *BCL10* is overexpressed and thought to form oligomers through its CARD domain without the need of upstream signals, leading to constitutive NF-κB activation. In MALT lymphomas with t(14;18)(q32;q21), *MALT1* is overexpressed and the oligomerisation and activation of MALT1 is thought to be dependent on BCL10. In MALT lymphomas with t(11;18)(q21;q21), the resulting API2–MALT1 fusion product is believed to self-oligomerise through the baculovirus IAP repeat (BIR) domain of the API2 molecule, therefore leading to constitutive NF-κB activity. Casp-L, caspase-like domain; CC, coiled coil; DD, death domain; GUK, guanylate-kinase-like domain; SH3, Src homology 3 domain; Ub, ubiquitin; Z, zinc finger. Reproduced from Isaacson and Du., 2004.[94]

1.1.5 Molecular basis of high grade transformation

The molecular events underlying the transition of an indolent low grade MALT lymphoma to a clinically aggressive DLBCL are ill-defined, but are of considerable interest due to the poor prognosis and limited treatment options associated with the latter disease. Although an accumulation of genetic aberrations clearly contributes to high grade transformation, many of the genetic abnormalities that appear to characterise the nodal form of DLBCL, such as BCL2 rearrangements, are absent in gDLBCL. Genetic alterations that have long been associated with histological transformation of MALT lymphoma include, complete inactivation of the *TP53* gene, hypermethylation of the promoter region of *TP15* and *TP16* as well as deletion of the *TP16* gene.[95-97] It has further been shown that in contrast to low grade MALT lymphoma, some gDLBCLs overexpress the BCL6 protein or harbour rearrangements of the *BCL6* locus on chromosome 3p27.[98-100] BCL6 acts as a transcriptional repressor and is required for GC formation and affinity maturation as well as being involved in cell-cycle arrest, inflammtion and apoptosis.[101-103]

In light of these early findings, Starostik et al.[84] recently compared the allelotypes of MALT lymphoma with gDLBCL via microsatellite screening and found amplification of the region 3p26.2-3p27 to be the most frequent aberration in both disease entities (Figure 1.6). The authors proposed that this aberration (harbouring the *BCL6* locus) may be a significant marker for high grade transformation of a proportion of MALT lymphomas.[84] The study further revealed that allelic imbalances in the 11q23-24 and 18q21 regions occurred with comparable frequencies in both low and high grade tumours.[84] Aberrations in regions 6q23.3-25 and 7q31 were present at a higher rate in gDLBCLs.[84] While, allelic imbalances in regions 2p16-21, 6p23 (*A20*), 12p12-13, 5q21, 9p21 (*TP16*), 13q14 (*RB*) and 17p13 (*TP53*) occurred exclusively in gDLCBL.[84] Although some of these deletion hotspots comprise known tumour suppressor genes (indicated in parentheses), the proportion of the allelic imbalances that are of biologic or clinical relevance to gDLBCL remains largely unclear.

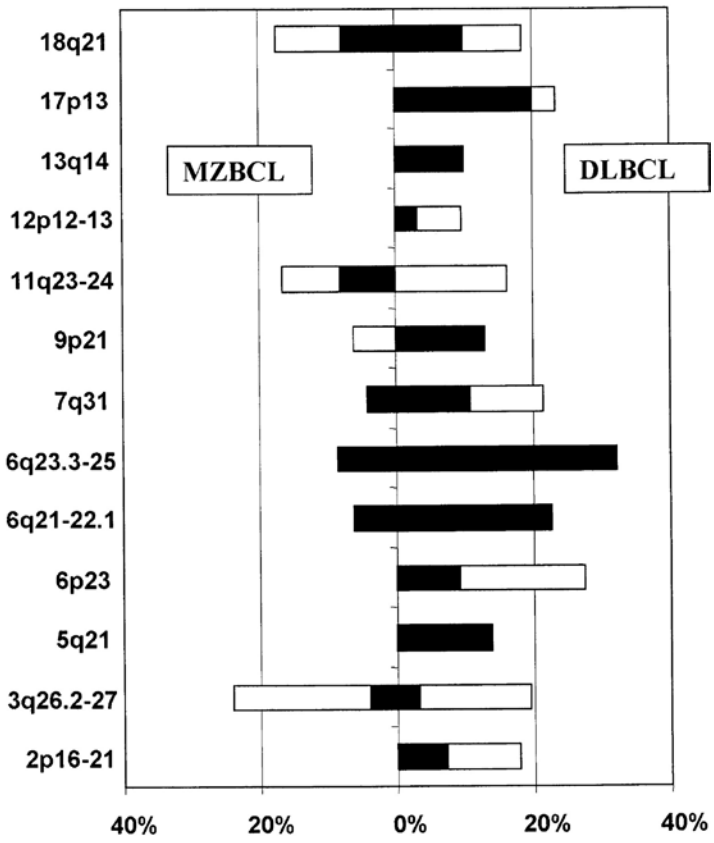

Figure 1.6 Comparison of the allelotypes of low grade MALT lymphoma and DLBCL. Frequency of allelic imbalance in individual regions is expressed as a bar diagram: open bar, amplification; black bar, deletion. The left-hand side shows results for MALT lymphoma (MZBCL); the right-hand side, results for DLBCL. Reproduced from Starostik et al., 2002.[84]

Disease progression and high grade transformation has also been linked to rearrangements of the *MYC* proto-oncogene. The *MYC* gene, located at chromosome band 8q24, encodes a basic helix-loop-helix transcription factor that modulates the expression of an expansive network of genes involved in cell proliferation, growth and apoptosis.[104] In stark contrast to their nodal counterparts, previous studies have reported that *MYC* rearrangements are reasonably frequent in gDLBCLs and therefore the deregulation of *MYC* expression might be an important factor in extranodal DLBCL. Van Krieken et al.[105] first noted in 1990, the presence of *MYC* rearrangements in 50% of high grade lymphomas of the stomach and the utter lack thereof in low grade lymphomas. This trend was swiftly reinforced by an independent study which found structural alterations of the *MYC* gene in 60% of DLBCLs derived from the stomach.[106] Likewise, Kramer et al.[107] reported that 60% of gDLBCLs versus 2% of nodal cases analysed were affected by MYC gene rearrangements. In contrast, a study by Peng et al. did not identify *MYC* rearrangements in any of the eleven cases of high grade MALT lymphoma examined, thereby arguing against

the potential involvement of *MYC* gene rearrangements in high grade lymphomagensis.[108] This group also compared the mutation frequency within the *MYC* gene between low and high grade MALT lymphoma but found no significant differences between the disease entities, indicating the mutations may have been acquired during early development of the tumours.[108]

The putative oncogene *FOXP1*, located at 3p13, codes for a member of the FOX family of transcription factors which are characterised by a common DNA-binding winged helix or forkhead domain. FOXP1 is known to play an important role in B-cell development, since *FOXP1*-null mice exhibit impaired early B-cell development with a block at the transition from pro-B to pre-B cells.[109] *FOXP1* was recently identified as a novel translocation partner of *IGH*, not only in MALT lymphoma but also in extranodal DLBCL.[110] The overall incidence of *FOXP1* gene rearrangements is, however, rare and a substantial number of t(3;14)(p13;q32)-negative cases have strong nuclear FOXP1 expression.[110, 111] This discrepancy suggests alternative mechanisms, other than underlying genetic alterations, as the responsible cause for FOXP1 deregulation. Interestingly, correlation studies have implicated FOXP1 deregulation in high grade transformation of MALT lymphoma to gDLBCL.[111, 112] In one study, Saegert et al.[112] reported the significant association of FOXP1-positivity with a five-year disease-free survival rate of 50% in patients compared to 100% in the FOXP1-negative group. In addition, the authors observed that all of the MALT lymphomas in the study which transformed into gDLBCL exhibited strong FOXP1 expression.[112] Despite the clinical implications of aberrant FOXP1 expression, no attempts have yet been made to precisely define the oncogenic mechanism(s) of this transcription factor.

A novel mechanism inducing genetic instability, termed aberrant somatic hypermutation (ASHM), has been described in the context of both nodal and gastric forms of DLBCL.[113, 114] Furthermore, this phenomenon appears to be associated with high grade transformation of chronic lymphocytic leukaemia (CLL).[115] Somatic hypermutation normally targets the 5′ end of *IGV* genes in GC B cells, but in some lymphomas it appears to misfire and aberrantly target multiple loci, including proto-oncogenes: *PIM1*, *PAX5*, *RHOH* and

MYC.[113] Sequence analysis of MALT lymphomas revealed that 75% of low grade MALT lymphomas and 100% of gDLBCLs contained mutations in one or more of these oncogenes.[107] Since higher frequencies of mutations were found in transformed MALT lymphoma, an active involvement of ASHM in high grade transformation has been speculated. By mutating important oncogenes, ASHM may therefore represent a major contributor to the pathogenesis and ultimate transformation of MALT lymphoma.

1.1.6 Animal models of *Helicobacter*-induced MALT lymphoma

The recognition of *H. pylori* as the etiological agent behind the formation of MALT lymphoma necessitated the development of an animal model to help elucidate the pathogenic mechanisms of this bacterium and facilitate the development of improved treatment strategies for the disease. An appropriate model was first achieved in 1995 in the laboratory of Adrian Lee following a series of experiments examining the effects of long-term colonisation by *Helicobacter* stains in BALB/c mice.[27] Infection of mice with a *Helicobacter* strain isolated from cats, *H. felis*, was found to induce acquisition of MALT and lead to the formation of gastric lymphomas after approximately 18-24 months (Figure 1.7).[27] The murine form of MALT lymphoma accurately mimics the histopathological features observed in human patients.

H. felis readily colonises murine stomachs at high densities and by 22 months post-infection up to 90% of animals develop LELs, a hallmark of MALT lymphoma in humans (Figure 1.7).[27] This model has been further validated by the demonstration of lymphoma regression following antibiotic eradication of *H. felis* from tumour-bearing mice.[116] Animals received a triple therapy consisting of bismuth subcitrate, metronidazole and tetracycline at 20 months post-infection; by four months post-treatment no lymphoid follicles were detected in the antibiotic-treated animals and only 10% of the animals had LELs.[116] Several studies have since highlighted that 10-25% of animals fail to respond to antimicrobial therapy, a result consistent with the remission rate in human patients.[11, 116] Interestingly, transcriptional profiling of whole stomach tissue from successfully treated mice revealed the long-term mucosal persistence of residual B cells.[11] This discrepancy between histologic and molecular remission is similar to the phenomenon observed in human patients, where the persistence of

monoclonal B-cells in the gastric mucosa occurs in roughly 50% of patients with complete histological remission.[49, 117] Mueller et al.[11] further reported that experimental reintroduction of *Helicobacter* in treated mice led to very rapid disease recurrence, with relapsed tumours exhibiting more aggressive behavior. Additional studies using this murine model have uncovered distinct transcriptional profiles that correlate with specific histopathological disease stages and allow accurate prediction of treatment outcome.[119]

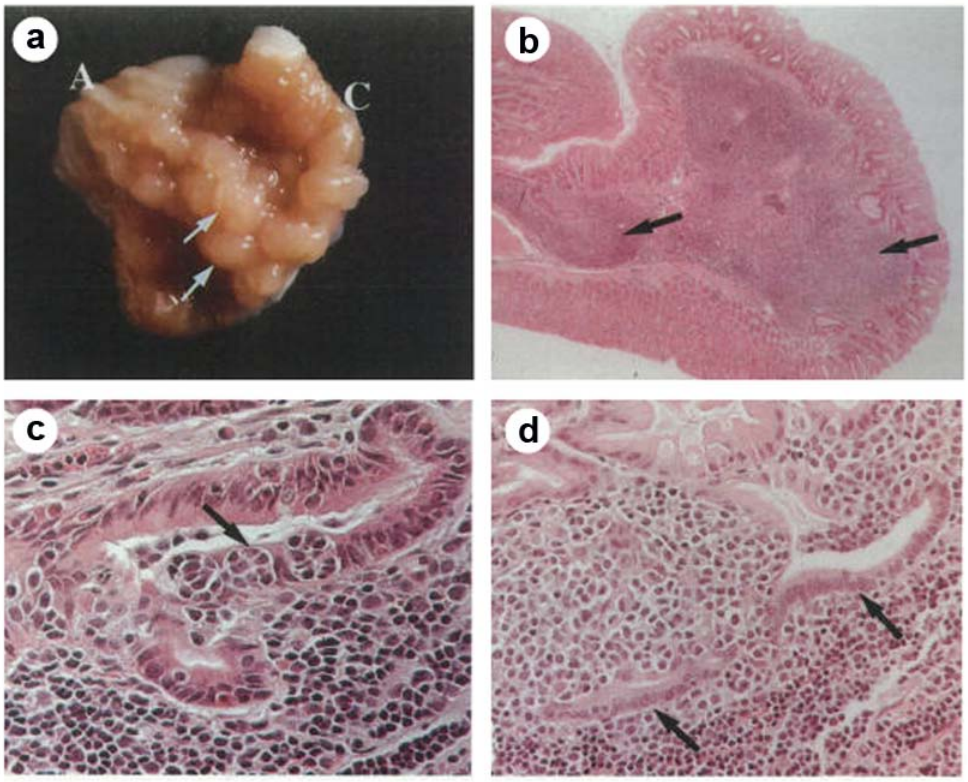

Figure 1.7 Histologic features of MALT lymphoma-like lesions in mice infected with *H. felis*. **a** Nodular gastric mucosa (arrows) of *H. felis*-infected BALB/c mouse; A = antrum; C = corpus mucosa. **b** Section through nodular mucosa showing lymphoid hyperplasia in the submucosa. Poorly demarcated germinal centres (arrows) are apparent within the lymphoid tissue (H&E). **c** Small aggregates of centrocyte-like cells (arrows) infiltrate hyperplastic foveolar epithelium in a relatively early lymphoepithelial lesion (LEL)(H&E). **d** Fully developed LEL lesion showing destruction of glandular and foveolar epithelium with a central epithelial remnant (arrows)(H&E). Reproduced from Enno et al., 1995.[27]

A comprehensive study comparing the virulence of *H. pylori*, *H. felis* and *H. heilmannii* in groups of BALB/c mice revealed the formation of lymphomas by all three species.[119] Although the appearance of the lesions were indistinguishable, *H. heilmannii* induced the most severe response in the highest proportion of infected animals.[119] However,

due to the difficulties involved in preparing *H. heilmanni* isolates, *H. felis* remains the preferred strain for experimental infection. Alternative animal models have also been sporadically employed to study MALT lymphoma. For instance, gastric MALT lymphomas, similar in morphology to those seen in humans, have been observed in ferrets infected with *H. mustelae*.[120]

1.2 MicroRNA and cancer

miRNAs represent an abundant class of small non-coding RNAs of ~18-25 nucleotides in length that negatively regulate eukaryotic gene expression at the post-transcriptional level. First identified in 1993 as regulators of developmental timing in *Caenorhabditis elegans*,[121] miRNAs are now recognized as central players in a plethora of important human biological processes such as development, differentiation, haematopoiesis, metabolism, cell cycle and apoptosis. Rapidly accumulating evidence implicates a direct role for miRNAs in cancer etiology and such studies have begun to significantly improve our understanding of carcinogenesis with important ramifications for disease diagnosis and treatment. Exploring the role of miRNAs in MALT lymphoma represents an exciting and thus far unprecedented avenue of research that could provide meaningful insight into the development and progression of this disease. Since direct studies on the role of miRNAs in MALT lymphoma are lacking, the following chapter will focus on the general connection between miRNA biology and tumourigenesis.

1.2.1 Biogenesis and mode of action

Human miRNA genes are non-randomly distributed in the genome as either single genes or, for a considerable part, in clusters of 2-7 genes. It is postulated that a given cluster is under the control of a single promoter and is transcribed as a polycistronic primary transcript from which the individual miRNAs are processed. While the majority of miRNA genes are situated in intergenic regions, a substantial proportion (~40%) are also located within introns or exons of both protein-coding and non-coding genes and may therefore be co-transcribed with their host-gene. In mammals, miRNAs are initially transcribed by RNA polymerase II as long primary transcripts characterised by hairpin structures (pri-miRNAs)

and subsequently processed in the nucleus by the RNAse III Drosha into miRNA precursors (pre-miRNAs) of ~70 nucleotides in length (Figure 1.8). These precursor molecules are transported to the cytoplasm by an Exportin-5 mediated mechanism. In the cytoplasm, another RNAse III enzyme, termed Dicer, converts pre-miRNAs to imperfect dsRNA duplexes of approximately 22 nucleotides (miRNA:miRNA*) that contain both the mature miRNA (miRNA*) and its complementary strand. The duplex is unwound by a RNA helicase and the mature miRNA strand is incorporated into the complex known as miRNA-containing RNA-induced silencing complex (RISC), whereas the remaining strand is most probably degraded.[122] As part of the RISC complex, mature miRNA is able to suppress target mRNA expression, mostly through interaction with the 3' untranslated region (UTR) leading to degradation or translation inhibition of the mRNA template (Figure 1.8) Although the degree of complementarity shared between miRNAs and their targets is an important factor, the exact molecular mechanisms that underlie post-transcriptional repression by miRNAs (reviewed in[123]) remain controversial.

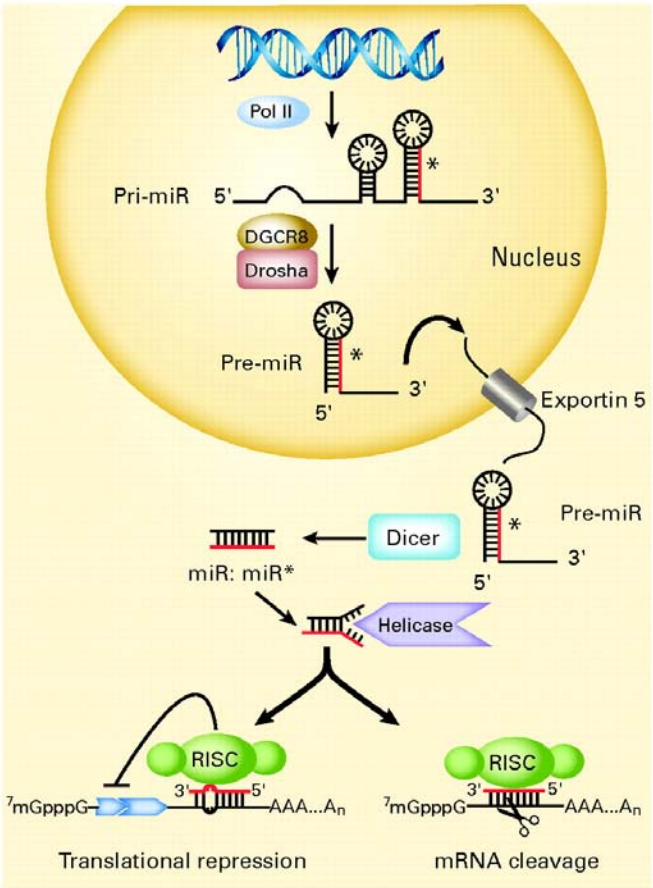

Fig 1.8 Biogenesis, maturation, and function of microRNAs (miRs). miRs are first transcribed by RNA polymerase II (Pol II) as long primary transcripts characterised by hairpin structures (pri-miRs) and processed in the nucleus into ~70-nucleotide-long pre-miRs by the microprocessor complex formed by RNAse III Drosha and its binding partner, DiGeorge critical region 8 (DGCR8). This precursor molecule is transported to the cytoplasm by Exportin 5. Pre-miRs are processed into miR:miR* duplexes of approximately 22 nucleotides by RNAse III Dicer. One strand of the miR:miR* duplex is then incorporated into the RNA-induced silencing complex (RISC), whereas the other strand is likely subjected to degradation. As part of this complex, the mature miR acts on its target by binding to the 3' untranslated region of target mRNAs leading to translational inhibition or mRNA cleavage, depending, at least in part, on the level of complementarity between the small RNA and its target sequence. Reproduced from Iorio and Croce et al., 2009.[124]

1.2.2 MicroRNA deregulation in cancer

1.2.2.1 Evidence for the role of microRNA in cancer

Cancer is a disease characterised by multi-step changes in the genome. To date, cancer research has largely focused on altered expression of protein coding genes. However, mounting evidence suggests that alterations of miRNAs also contribute to tumourigenesis. Direct evidence for the involvement of miRNAs in cancer was first reported by Calin et al.[125] who found two miRNAs, *miR-15a* and *miR-16-1*, clustered at chromosome 13q14, a region deleted in more than 50% of CLL cases. These miRNAs were found to target *BCL2*, an oncogene commonly overexpressed in CLL patients, thus establishing a tumour suppressive function for the *miR-15a/miR-16-1* cluster.[126] Several groups have since described aberrant miRNA expression patterns in a wide array of cancers including various haematological malignancies[127-129] and solid tumours.[130-133] The advent of high-throughput miRNA quantification technologies, such as microarray[134, 135] and real-time PCR-based assays[136, 137] have permitted the identification of global miRNA profiles of whole cancer genomes including distinct miRNA expression signatures associated with the diagnosis, prognosis and response to therapy of several tumour types.[127, 130, 131, 138-140] Surprisingly, it has become evident that despite the much smaller number of miRNA genes (~700) compared to protein coding genes (~22,000), the miRNome is a superior means for classifying cancer type and tumour stage, compared to the mRNA expression profile.[130-132] In cancer, miRNAs have the propensity to function as either oncogenes or tumour suppressors, depending on their target mRNA. Although some miRNAs are overexpressed, the majority are downregulated in cancer with respect to normal tissue.[130, 140, 141] This phenomenon has led to the hypothesis that global miRNA expression reflects the state of cellular differentiation[130] and therefore the widespread repression of miRNA may represent a hallmark of human cancer.

1.2.2.2 Mechanisms of microRNA deregulation

In normal development, miRNA expression is tightly regulated, both spatially and temporally in a highly tissue-specific manner.[142] Aberrant expression of miRNAs, indicative of a disease state, can be caused by various mechanisms including deletions, amplifications

or mutations involving miRNA loci, epigenetic factors, transcriptional deregulation and defects in miRNA processing (Figure 1.9).

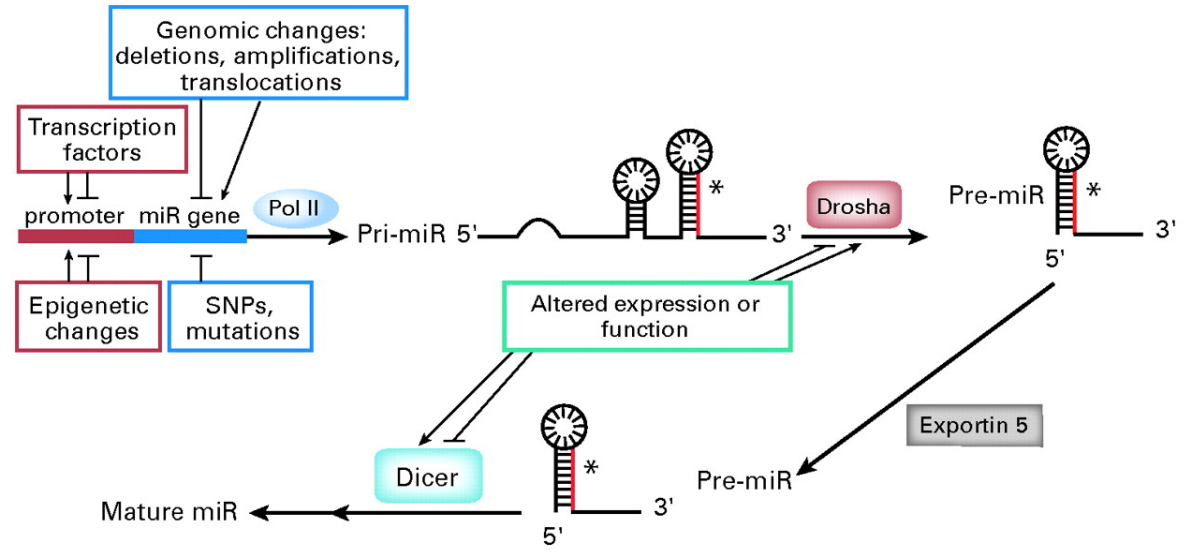

Fig 1.9. **Various mechanisms of microRNA (miR) regulation.** Deregulated miR expression observed in cancer can be caused by chromosomal abnormalities, mutations, polymorphisms (SNPs), transcriptional deregulation, defective processing and epigenetic changes. Reproduced from Iorio and Croce et al., 2009[124]

Genomic abnormalities

Cancer is often accompanied by chromosomal abnormalities and a significant proportion of human miRNAs appear to reside in regions frequently rearranged in human cancer (Table 1.3)[143-146] and in mice miRNA genes are often located at cancer susceptibility loci.[147] Emerging evidence suggests that germ-line and/or somatic mutations and polymorphisms of miRNAs might also contribute to deregulation of mature miRNAs.[148] For example, a G to A germ line mutation 19 nucleotides downstream of the pre-*let-7e* miRNA detected in human tumours, led to a significant reduction of its expression.[149] Likewise, a germ line mutation (C to T) situated seven nucleotides downstream of the *pre-miR-16-1* sequence was identified in CLL patients and found to significantly reduce the expression of both *miR-15a* and *miR-16-1*.[148] Mutations can also affect the target mRNA site; this concept was highlighted by a discovery in papillary thyroid carcinoma, where 50% of patients harboured point mutations within the *KIT* oncogene precisely mapping to the 3'UTR binding

domain for *miR-221* and *miR-222*.[150] Felli et al.[151] previously described the ability of *miR-221* and *miR-222* to downregulate the *KIT* oncogene, implying that these miRNAs contribute to cancer progression by regulating KIT expression.

Table 1.3 Examples of microRNAs located in deleted, amplified or breakpoint regions commonly involved in human cancers. Adapted from[143-146].

Chromosomal rearrangement	MicroRNA	Malignancy
1p36-*D*	*mir-34a*	Neuroblastoma
3p21–21-*D*	*let-7g/mir-135-1*	Lung, breast cancer
3p21-*D*	*mir-26a*	Epithelial cancer
3p23–21-*D*	*mir-26a; mir-138-1*	Nasopharyngeal cancer
5q32-*D*	*mir-145/mir-143*	Myelodysplastic syndrome
7q32-*D*	*miR-29b-1/miR-29a*	Myelodysplastic syndrome, acute myeloid leukemia
9q22-*D*	*mir-24-1/mir-27b/mir-23b;let-7a-1/let-7f-1/let-7d*	Urothelial cancer
9q33-*D*	*mir-123*	NSCLC
11q23–q24-*D*	*mir-34b/mir-34c;mir-125b-1/let-7a-2/mir-100*	Breast, lung cancer, ovary, cervical cancer
13q14-*D*	*mir-15a/mir-16-1*	Chronic lymphocytic leukemia
13q32-33-*A*	*mir-17/mir-18/mir-19a/mir-20/mir-19b-1/mir-92-1*	Follicular lymphoma
17p13-*D*	*mir-22; mir-132; mir-122;mir-195*	HCC, Lung cancer
17q22-t(8;17)	*mir-142*	Prolymphocytic leukemia
17q23-*A*	*mir-21*	Neuroblastoma, breast cancer
20q13-*A*	*mir-297-3*	Colon cancer
21q11-*D*	*mir-99a/let-7c/mir-125b*	Lung cancer

D, deleted region; *A*, amplified region; NSCLC, non-small-cell lung cancer; HCC, hepatoceullular carcinoma; OG, oncogene; TS, tumour suppressor; miRNAs in a cluster are seperated by a slash.

Epigenetic alterations

Epigenetic events, such as DNA methylation and histone modification, have also been linked to the deregulation of miRNA expression in cancer. *In silico* analyses has revealed that a significant proportion of miRNA gene promoters contain a CpG island.[152, 153] In addition, several groups have shown that DNA-demethylating agents and/or histone deactylase (HDAC) inhibitors upregulate the expression of miRNAs in cancer cells.[139, 154, 155]

A representative example is provided by the work of Saito et al.[154] wherein 17 out of 313 human miRNAs analysed were upregulated more than three-fold in bladder cancer cells upon treatment with chromatin modifying drugs. Of these miRNAs, *miR-127* was the most differentially expressed and was found to be embedded in a CpG island.[154] It was further shown that *miR-127* acts as a tumour suppressor through BCL6 repression.[154] On the other hand, miRNAs themselves can regulate DNA methylation by targeting components of the epigenetic machinery. For instance, it has been shown in both lung cancer and acute myloid leukaemia that *miR-29* directly targets *DNMT3A* and *-3B*.[156, 157] Enforced expression of *miR-29* in lung cancer cell lines restored normal patterns of DNA methylation, induced re-expression of silenced tumour suppressor genes and inhibited tumourigenicity *in vitro* and *in vivo*.[156]

Transcriptional regulation

Transcriptional regulation is a crucial feature of the fine tuning of miRNA expression in cells. Although many miRNA-transcription factor relationships have been discovered in cancer, much attention has focused on the role of the proto-oncogene *MYC* in the regulation of miRNA expression. Deregulated expression or function of MYC is one of the most common features of human cancer.[158] MYC was initially found to directly transactivate an oncogenic group of miRNAs known as the *miR-17–92* cluster.[159] The *miR-17-92* cluster, which consists of six miRNAs co-transcribed from a locus on chromosome 13, is frequently amplified in B-cell lymphomas and solid malignancies.[160, 161] Conversely, studies have revealed that *MYC* activation leads to widespread repression of a large group of miRNAs, several of which are known tumour suppressors such as *let-7* family members, *miR-15a/16-1* and *miR-34a*.[162, 163] Chang et al.[162] revealed that much of this repression is likely to be a direct result of MYC binding to miRNA promoters. Reintroduction of MYC-repressed miRNAs was shown to markedly suppress tumourigenesis *in vivo*, supporting a central role for miRNA repression in the complex network of MYC-induced tumourigenesis.[162]

Defects in the miRNA biogenesis pathway

Factors affecting miRNA processing have also been implicated in tumourigenesis. A comprehensive study headed by Kumar et al.[164] demonstrated that impaired miRNA biogenesis promotes tumourigenesis *in vivo* though deregulation of specific oncogenes. Drosha and Dicer serve as key regulatory proteins in the miRNA processing pathway and their alterations have been observed in a number of cancers.[164-166] Karube et al.[165] found that a significant proportion of lung cancers have reduced expression levels of Dicer and this downregulation was associated with shortened postoperative survival. In breast cancer, Dicer expression is significantly downregulated in the more aggressive tumour types.[166] Corresponding to this, abnormalities of the long arm of chromsome 14, where *Dicer* is situated, has been reported in various cancers.[167-170] Most recently it was shown that low Dicer and Drosha expression levels are significantly associated with survival in patients with ovarian cancer.[171] Altogether, the current evidence suggests that defective miRNA processing may promote tumourigensis.

2 AIM OF MY STUDIES

Gastric MALT lymphoma provides an outstanding model for studying multiple aspects of lymphoma development. In order to broadly improve our understanding of the pathogenesis of this disease at the molecular level, three main objectives were pursued in the course of my studies.

- **Determination of the specificity of gastric MALT lymphoma surface immunoglobulin.** To address the issue of antigen receptor specificity we aimed to produce a panel of both human and murine recombinant MALT lymphoma-derived, surface-exposed tumour immunoglobulins. An ELISA approach could then be implemented to perform a comprehensive screen of antigen reactivity.

- **Elucidation of the role of infiltrating T cells in MALT lymphoma.**
  In this study, the composition of T-cell subsets was assessed in order to determine the presence of regulatory T cells in the MALT lymphoma environment. We further endeavoured to address the impact of infiltrating T cells on tumour B-cell proliferation *in vitro* and on tumour development *in vivo*.

- **Investigation of miRNA deregulation in MALT lymphomagensis.**
  Initially we set out to determine the unique miRNA expression profile associated with the development and progression of MALT lymphoma. Subsequently, we aimed to identify relevant tumour suppressor miRNAs in both low and high grade MALT lymphoma. Possible mechanisms causing deregulation of miRNA expression were also addressed in this project.

3 RESULTS

Short summaries and reprints of publications and manuscripts describing the results obtained during my PhD.

3.1 Gastric MALT lymphoma B-cells express polyreactive, somatically mutated immunoglobulins

Authors: Vanessa J. Craig, Isabelle Arnold, Christiane Gerke, Minh Q. Huynh, Thomas Wundisch, Andreas Neubauer, Christoph Renner, Stanley Falkow and Anne Mueller

Journal: Blood, 21 January 2010, Vol. 115, No. 3, pp. 581-591.
DOI 10.1182/blood-2009-06-228015.

Summary: This publication describes our efforts to characterise and determine the specificity of MALT lymphoma surface immunoglobulin. A panel of murine and human MALT lymphoma-derived immunoglobulins were cloned and recombinantly expressed. Sequence analysis revealed the majority of murine tumours were monoclonal, had undergone somatic hypermutation and more than half of all tumours showed evidence of intraclonal variation and positive and/or negative selective pressure. The recombinantly expressed MALT lymphoma antibodies exhibited a polyreactive binding profile in a series of ELISA assays. Auxiliary to this, a strong bias towards the use of V_H gene segments often linked to auto- and/or polyreactive antibodies was found. Taken together, the results suggest that MALT lymphoma development may be facilitated by an array of local self and foreign antigens that provide direct antigenic stimulation of the tumour cells via their B-cell receptor.

LYMPHOID NEOPLASIA

Gastric MALT lymphoma B cells express polyreactive, somatically mutated immunoglobulins

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Gastric B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) arises against a background of chronic inflammation caused by persistent *Helicobacter pylori* infection. The clinical and histopathologic features of the human tumor can be reproduced by *Helicobacter* infection of BALB/c mice. In this study, we have analyzed the antibody sequences and antigen specificity of a panel of murine and human MALT lymphoma–derived antibodies. We find that a majority of tumors in patients as well as experimentally infected mice are monoclonal. The tumor immunoglobulin heavy chain genes have undergone somatic hypermutation, and approximately half of all tumors show evidence of intraclonal variation and positive and/or negative selective pressure. Recombinantly expressed MALT lymphoma antibodies bind with intermediate affinity to various unrelated self- and foreign antigens, including *Helicobacter* sonicate, immunoglobulin G (IgG), DNA, and stomach extract; antigen binding is blocked in a dose-dependent manner in competitive enzyme-linked immunosorbent assays. A strong bias toward the use of V_H gene segments previously linked to autoantibodies and/or polyreactive antibodies in B-cell malignancies or autoimmune pathologies supports the experimental finding of polyreactivity. Our results suggest that MALT lymphoma development may be facilitated by an array of local self- and foreign antigens, providing direct antigenic stimulation of the tumor cells via their B-cell receptor. (Blood. 2010;115:581-591)

Introduction

Gastric mucosa-associated lymphoid tissue (MALT) B-cell lymphoma develops in the context of long-term infection with the Gram-negative gastric bacterium *Helicobacter pylori*.[1-3] Persistent infection with *H pylori* causes chronic gastritis that, in some people, can develop into more organized gastric mucosa-associated lymphoid tissue (MALT) with histologic similarity to the Peyer patches of the small intestine.[4] The disease progresses when individual malignant clones grow out, displace the benign lymphoid tissue, and ultimately form the lymphoepithelial lesions that are a hallmark of MALT lymphoma.[5,6] In its early stages, gastric MALT lymphoma is believed to be an antigen-dependent disease; *H pylori* infection is detectable in a large majority of cases.[1,3,7] Eradication therapy induces tumor regression in approximately 75% of patients at this stage.[8,9] Early-stage low-grade MALT lymphoma is generally considered an indolent tumor due to its slow growth, low proliferation rates, and minimal propensity for spreading. At later stages, however, the tumors can eventually undergo high-grade transformation or acquire one of several known characteristic chromosomal translocations, thereby rendering the lymphoma independent of antigen exposure and refractory to *H pylori* eradication therapy.

In contrast to cases with chromosomal rearrangements, which grow autonomously due to constitutive activation of the nuclear factor κB (NF-κB) signaling pathway brought about by overexpression of MALT-1, B-cell leukemia 10 (Bcl-10), or production of the Baculovirus IAP repeat-containing 3 (API2)–MALT1 fusion protein (reviewed in Isaacson and Du),[5] little is known about the pathogenesis of early MALT lymphoma. Several studies have implicated the abundant population of tumor-infiltrating T cells in providing growth signals to tumor B cells.[10-12] In one study, depletion of T cells was shown to abrogate the proliferation of explanted MALT lymphoma cultures.[12] We and others have reported that MALT lymphomas express high levels of interleukin-4 (IL-4) and other T helper 2 cytokines in vivo, supporting a role for T helper 2–polarized T-helper responses in early MALT lymphomagenesis.[10,11]

An alternative possibility is that early stage MALT lymphoma B cells receive signals via antigenic stimulation through their B-cell receptor (BCR), which would lead to NF-κB activation, survival, and proliferation. Indeed, MALT lymphoma cells carry functional, rearranged, and somatically mutated immunoglobulin genes on their surface.[13,14] Sequence analysis of the V_H genes suggests that the tumor cells have undergone positive selection in germinal centers.[14-16] Intraclonal variation caused by ongoing somatic mutation and/or replacement of a part of the variable heavy segment (receptor revision) has been reported.[15-17] Despite these clear results, the search for a target antigen has proven difficult and has yielded controversial results, either providing evidence for reactivity toward certain structures of normal human tissues (follicular dendritic cells, venules, epithelial cells, connective tissue)[18,19] or human immunoglobulin G (IgG),[13] or not identifying any target antigens at all.[20]

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We and others have shown earlier that long-term, persistent infection of the BALB/c strain of mice with *H pylori* or its close relatives *Helicobacter felis* and *Helicobacter heilmannii* results in the reproducible induction of lesions that clinically and histologically resemble human MALT lymphoma.[10,21-23] These lesions are slow growing, spread only infrequently (usually to the spleen), and rarely transform to high-grade lymphoma.[10,22] The tumors further recapitulate the human disease in that they regress in a majority of mice upon eradication of the infection,[22] but recur rapidly upon reintroduction of the bacteria, revealing gastric persistence of the tumor clone despite complete histologic remission.[10]

Using this experimental mouse model as well as human MALT lymphoma biopsy material, we deduced and compared the immunoglobulin sequences of 20 murine and 7 human tumors. Based on the monoclonal Ig sequences, we generated a comprehensive panel of recombinant antibodies expressed as soluble IgG of matching heavy and light chains and tested their reactivity toward various self- and foreign antigens. The tumor antibodies, which were functional and somatically hypermutated and in some cases showed evidence of positive and/or negative selection, surprisingly reacted with roughly equal affinity with both gastric self- and *Helicobacter*-derived foreign antigens. This pattern of reactivity is consistent with a diagnosis of polyreactivity, and could further be linked to a strongly biased use of specific V_H family gene segments in both the murine and the human system.

Methods

Animal experimentation, tumor cell cultures, and immunophenotyping

Specific pathogen-free female BALB/c mice were infected orally at 6 weeks of age with 3 consecutive doses of approximately 5×10^7 *H felis* (CS1, ATCC 49179). All animal experiments were approved by Stanford University's Institutional Animal Care and Use Committee. Upon killing, the stomachs were removed and opened along the lesser curvature; macroscopically visible tumors were dissected. Single-cell suspensions were generated and cultured in RPMI supplemented with 10% fetal calf serum (FCS) and antibiotics. Where appropriate, 10 μg/mL *Helicobacter* lysate was used. Tumor cell proliferation was quantified by [^{3}H]-thymidine incorporation assay, or by bromodeoxyuridine (BrdU) incorporation followed by fluorescence-activated cell sorting analysis using a fluorescent in situ cell proliferation kit (Roche Diagnostics) The following antibodies were used for immunophenotyping: anti-CD19 (monoclonal antibody [mAb] clone 6D5; Abcam), anti-B220 (mAb clone RA3-6B2, BD Pharmingen), anti-IgM (polyclonal goat, no. 1020; Southern Biotech), anti-IgG (polyclonal goat, no. 1030; Southern Biotech), anti-CD3 (mAb clone 145-2C11; BD Pharmingen), anti-CD11c (mAb clone N418; AbD Serotec), anti–proliferating cell nuclear antigen (PCNA; mAb clone PC10; Zymed Labs), and anti-CD11b (mAb clone M1/70.15; AbD Serotec). Flow cytometry was performed on a CyanADP instrument (Dako).

Patient material

Human patient material was obtained from 7 patients with gastric MALT lymphoma who were part of a previously published study conducted at Philipps-University Marburg.[24] All tumors were diagnosed as *H pylori*–positive low-grade gastric MALT lymphomas and all were negative for the translocation t(11;18)(q21:q21). The 5 cases of chronic lymphocytic leukemia (CLL), diagnosed according to standard criteria, were described in previous studies[25,26]; all 5 tumor-derived IgV_H sequences had been determined previously to be somatically mutated.

RNA extraction, V_H and V_L gene amplification, and cloning

RNA was isolated from the murine tumor cell suspensions and from patient biopsy material using the RNeasy kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized using oligo-dT primers and SuperScript III reverse transcriptase (Invitrogen). The human Ig heavy and light chain sequences were obtained using Ig framework-specific primers for all human variable heavy and light chain framework 1 families in combination with primers specific for all human J segments, as specified by Marks et al.[27] The obtained variable heavy and light chain products were cloned into expression vectors in frame with a 5′ leader peptide and a 3′ human IgG1 or kappa light chain constant domain. The amplification and cloning of murine Ig variable heavy and light chain sequences was performed accordingly.

Sequence analysis

Sequencing of heavy and light chain variable regions was performed by Microsynth. The obtained sequences were aligned to germline sequences from the IGMT database (international ImMunoGeneTics database, http://www.imgt.org).[28] Tumors were defined as clonal if identical or near identical V_H sequences were obtained from 2 independent polymerase chain reactions. Somatic hypermutation and intraclonal variation of IgV_H genes was assessed as described by Lossos et al[29]; V_H gene sequences that deviated by more than 2% from the corresponding germline gene sequence were defined as somatically hypermutated. The degree of intraclonal diversity of IgV_H genes was defined as follows: an unconfirmed mutation is a substitution mutation observed in only one of the V_H gene clones from the same tumor specimen; a confirmed mutation is a mutation observed in more than one V_H clone from the same tumor specimen.[29] The role of antigen selection in shaping MALT lymphoma immunoglobulins was assessed by calculating and comparing the ratios of replacement (R) to silent (S) mutations in the framework (FR) and complementary determining regions (CDRs) of the somatically mutated Ig heavy chain genes. Positive selection was diagnosed if the R/S ratios of mutations in the CDRs were greater than 2.9; negative selection pressure by antigen was postulated if the R/S ratios in the FR regions were lower than 2.9.[30] The probability that an excess or scarcity of replacement mutations in V_H CDRs or framework regions occurred by chance was calculated by a multinomial distribution model.[31] A P value less than .05 was considered statistically significant.

Expression and purification of recombinant antibodies

293T cells were cultured in Opti-MEM I reduced Serum Media (Invitrogen) supplemented with GlutaMAX (Invitrogen). Cells were transfected by calcium phosphate coprecipitation with equimass amounts of the heavy and light chain vector and the supernatant was harvested after 7 days. Recombinant antibodies were purified from culture supernatants on a 1-mL HiTrap Protein G Sepharose column (Amersham Pharmacia Biotech) and dialyzed against phosphate-buffered saline (PBS).

Preparation of *H felis* antigens, murine stomach extract, and AGS cell extract

H felis and *H pylori* were grown as described.[21] Sonicate was prepared by harvesting cells in PBS and the cells were disrupted by sonication (Bandelin Sonopuls GM 70; Bandelin). For preparation of gastric mucosal extract, the mucosa was scraped from wild-type BALB/c mice and lysed in lysis buffer (10% glycerol, 1% Triton X-100, 100 nM NaCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.4], and 1mM sodium vanadate). For preparation of human stomach cell extract, human gastric carcinoma cells (AGS) were lysed with the same buffer. The protein content of all antigen preparations was determined by Bicinchoninic acid protein assay (Thermo Scientific) and aliquots were stored at −70°C until used.

Enzyme-linked immunosorbent assays

Duplicate wells of Maxisorp micotiter plates (Nunc) were coated with various antigens. Single-stranded DNA (ssDNA) and double-stranded DNA

(dsDNA) were coated at 100 μg/mL in PBS. All other antigens tested were coated at 5 μg/mL each in PBS. After washing with PBS-0.5% (vol/vol) Tween-20, the wells were blocked with 2% bovine serum albumin (BSA) in PBS for 2 hours. All antibodies were incubated overnight at 0.1, 1, and 10 μg/mL at 4°C. After washing, wells were incubated for 1 hour with either peroxidase-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories) or goat peroxidase-conjugated anti–human IgG (Abcam). Enzyme activity was detected by incubation with tetramethylbenzidine (Sigma-Aldrich). Rheumatoid factor activity of murine and human antibodies was determined using the mouse rheumatoid factor Ig enzyme-linked immunosorbent assay (ELISA) kit (Alpha Diagnostic International) and the Aeskulisa Human Rf-AGM kit (Aesku Diagnostics), respectively. The dissociation constants (K_d) were calculated according to Friguet et al.[32]

Results

Induction of *Helicobacter*-dependent gastric MALT lymphoma in a BALB/c mouse model and ex vivo culturing of tumor cell suspensions

To induce the formation of *Helicobacter*-dependent gastric MALT lymphoma, 18 female BALB/c mice were infected with *H felis* for 18 months; 7 additional age-matched females served as uninfected controls. Sixteen of the 18 infected, but none of the uninfected mice, had developed gastric MALT lymphoma at the time of killing. The tumors were macroscopically visible as nodules ranging from 1 to 2 mm in diameter. They were positive for the B-cell marker B220 and had proliferative indices of approximately 30% as determined by PCNA staining (Figure 1A-B). The tumors had formed predominantly at the forestomach/corpus junction. A total of 20 macroscopically discernible tumors were dissected and single-cell suspensions were generated of individual tumors. These were subjected to Ig sequence analysis (all 20 tumors; isolated from 10 individual mice), immunophenotyping (a subset of 14 tumors, from the same 10 mice), and/or ex vivo culturing (9 tumors from 8 mice).

In the tumor cell suspension cultures, an average of 64% of all leukocytes were $CD19^+$ B cells (Figure 1C); most of the remaining leukocytes stained positive for CD3 (Figure 1C). Only a minor fraction was positive for CD11b or CD11c (1.5% on average; data not shown). The suspensions generally contained less than 15% nonleukocyte cells. Although all tumor cell suspensions could be kept alive for 5 days in standard cell culture media, only cultures to which *Helicobacter* lysate had been added proliferated within this time frame, as determined by $[^3H]$-thymidine (Figure 1D) or BrdU incorporation (Figure 1E). Addition of *Helicobacter* lysate induced an on average 4-fold increase in $[^3H]$-thymidine incorporation compared with the corresponding unstimulated culture (Figure 1D); BrdU-positive cells increased from approximately 2.5% in unstimulated cultures to approximately 3.8% in stimulated cultures (Figure 1E). Immunophenotyping of BrdU-positive cells further revealed that the proliferating population in the cultures consisted predominantly of $CD19^+$ B cells (average: 83%; Figure 1F), with T cells accounting for the remaining proliferative subset. In conclusion, murine MALT lymphoma B cells not only are antigen dependent in vivo as we and others have shown previously by inducing tumor regression through *Helicobacter* eradication therapy,[10,22] but also retain their dependence on *Helicobacter* antigen ex vivo.

Immunophenotyping of murine MALT lymphomas

Human MALT lymphomas are known to express surface IgM and pan-B-cell markers (CD19, CD20, CD79a) and the marginal zone markers CD35 and CD21, although are negative for CD5, CD10, CD23, and cyclin D1.[33] Because we aimed to analyze the antibody sequences and specificity of our murine tumors, we flow cytometrically determined the surface IgM and IgG expression of 14 tumor cell suspensions (Figure 2). Indeed, the majority of $CD19^+$, $B220^+$ B cells in every tumor showed high expression of IgM (average: 90%); a minority expressed high IgG (average: 1.5%) and the rest expressed only low levels of either Ig (Figure 2).

Sequence analysis of murine MALT lymphoma immunoglobulins

To determine the clonal status of a panel of 20 murine MALT lymphomas, the corresponding cDNAs were generated and subjected to several rounds of cloning and sequencing of the IgV_H genes. (All sequences reported in this paper have been deposited in the GenBank database; accession nos. GQ856044-GQ856073.[34]) Tumors were pronounced monoclonal if more than half of all obtained IgV_H sequences were identical or near identical in 2 independent rounds of sequencing. Clear monoclonal status was demonstrated for 11 lymphomas (55% of cases), whereas 1 lymphoma appeared biclonal and the remaining 8 tumors were classified as polyclonal. None of the monoclonal sequences harbored internal stop codons, suggesting that murine MALT lymphoma B cells express potentially functional surface IgM.

The 11 clonal IgV_H sequences were further analyzed for evidence of somatic hypermutation and intraclonal variation (Table 1). Germline genes with the highest homology to the consensus tumor IgV_H sequence were identified (supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article); with the exception of 2 cases, all IgV_H genes analyzed displayed somatic mutation, that is, they deviated from the most closely related germline sequence in more than 2% of positions, with the average homology being 92.2% (Table 1, supplemental Figure 1). This result is in line with previous studies reporting somatic hypermutation of human MALT lymphoma IgV_H.[14-16] Four IgV_H sequences were found to be positively selected by antigen, that is, the ratios of replacement/silent mutations in their CDRs were higher than would be expected if mutations had occurred by chance alone without selective forces (based on a cutoff ratio of > 2.9[30]; Table 1). Six sequences demonstrated the presence of negative selection pressure by antigen, that is, the replacement/silent mutation ratios in their FR regions were lower than expected by chance alone. Two sequences showed evidence of both negative and positive selection. Analysis of antigen selection using the more stringent multinomial model[29,31] identified only 3 sequences with significant positive selection and one with significant negative selection (Table 1). Previous studies examining antigenic selection of human IgV_H have mostly reported evidence of positive selective pressure,[14-16] suggesting that MALT lymphoma B cells have undergone germinal center reactions and affinity maturation.

To evaluate the presence of ongoing mutation, the 11 clonal sequences were examined by comparing at least 8 and up to 22 molecular clones from each tumor (supplemental Figure 2). Five of the somatically mutated clonal V_H gene isolates did not show intraclonal heterogeneity (Table 1), whereas the remaining 6 tumors, including the 2 with unmutated IgV_H sequences, harbored confirmed mutations and therefore showed evidence of intraclonal variation (Table 1, supplemental Figure 2). The average length of the CDR3 region for the 11 monoclonal tumor immunoglobulins was 12.5 amino acids, which is longer than the average

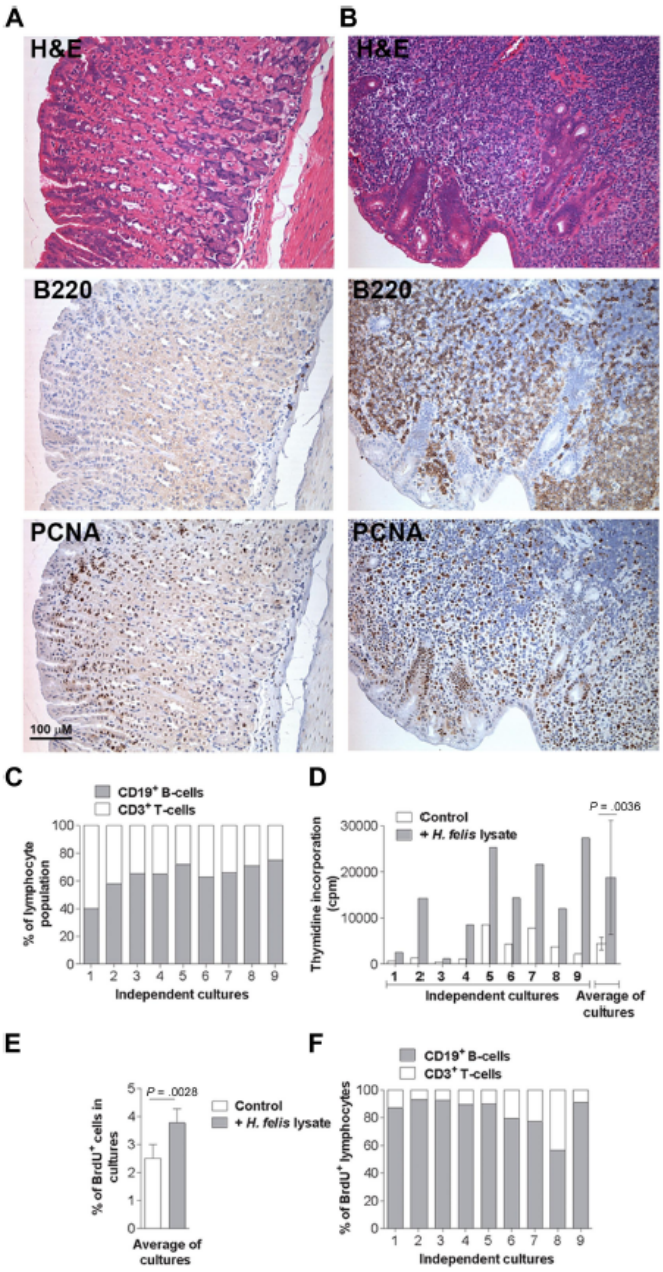

Figure 1. Murine MALT lymphoma cells proliferate in response to *Helicobacter* antigen. (A-B) Histopathology of murine mucosa-associated lymphoid tissue (MALT) lymphoma. Consecutive paraffin sections were stained with hematoxylin and eosin (H&E), the B-cell marker B220, and the proliferation marker PCNA; representative sections are shown for an unaffected area (A) and a lymphoma (B) from the same stomach. (C-F) Single-cell suspensions of 9 murine MALT lymphomas were cultured in the presence or absence of 10 μg/mL *Helicobacter felis* lysate. The proportion of $CD19^+$ B cells and $CD3^+$ T cells was determined flow cytometrically for each culture (C). Proliferation was determined by $[^3H]$-thymidine incorporation (D) or BrdU incorporation (E) after 5 days of culturing. Individual cultures and/or averages of all cultures are shown. Error bars represent SD. (F) The $BrdU^+$ cells are mostly B and T cells.

length of 8.5 amino acids usually found in a normal murine splenic B-cell repertoire (Table 1).[35] V_H gene family use was found to be strongly biased toward V_H1 (n = 7) and V_H14 (n = 4). Of the 12 known murine V_H gene families, the V_H1 gene family accounts for 28% of the antibody repertoire in BALB/c splenocytes; the V_H14 family in contrast is rarely used in BALB/c mice.

Antigen reactivity of recombinant murine MALT lymphoma–derived antibodies

To investigate the antigen specificity of murine MALT lymphoma Ig, the corresponding light chains of 5 of the clonal heavy chains were identified. In cases with ongoing mutation, the IgV_H sequence most closely matching the consensus sequence was chosen. Paired heavy and light chains were expressed recombinantly in 293T cells and purified by affinity chromatography. We deliberately selected a mixed panel of mutated and unmutated, antigen selected and unselected IgV_H sequences (mu Abs 2, 3 8, 10, and 11). A series of ELISAs were performed to systematically screen Ig reactivity toward a broad range of antigens. Possible autoreactivity was assessed using murine stomach extract; antinuclear activity was examined using both ssDNA and dsDNA, and rheumatoid factor activity was assessed by IgG binding; the foreign antigens tested included *H felis* and *Escherichia coli* sonicate and purified *E coli* lipopolysaccharide (LPS). BSA was used as a negative control. The

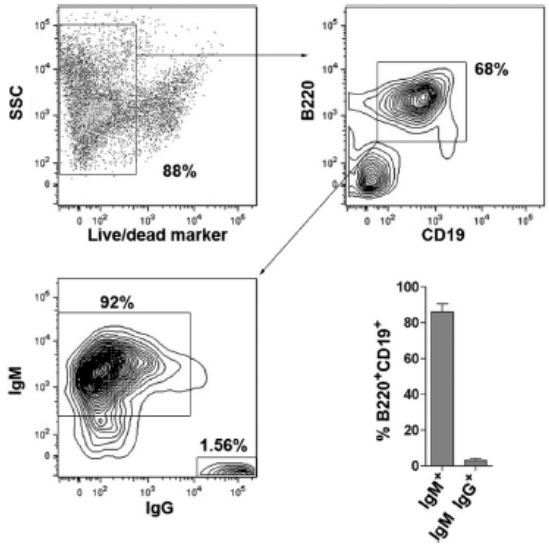

Figure 2. Murine MALT lymphoma cells express B220 and CD19 and are surface IgM positive. Fourteen independent single-cell suspension cultures of murine MALT lymphomas were analyzed flow cytometrically with respect to B220, CD19, and IgM/IgG expression. Results from a representative culture as well as the averages of all 14 cultures are shown. Error bars represent SEM.

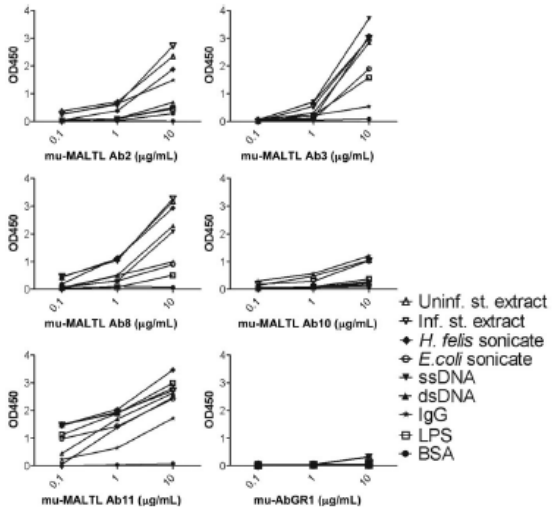

Figure 3. Recombinant murine MALT lymphoma–derived antibodies are polyreactive. Three concentrations (0.1, 1, and 10 μg/mL) of 5 murine antibodies cloned from the tumors of 5 independent mice and recombinantly expressed in 293T cells were tested by ELISA for reactivity toward BSA, LPS, IgG, dsDNA, ssDNA, *Escherichia coli*, or *Helicobacter felis* sonicate and protein that was extracted from the mucosa of BALB/c stomachs (either infected with *H felis* or uninfected). Antibodies 2, 3, 8, and 11 display strong reactivity toward the majority of targets, whereas Ab10 has comparatively low affinity. A monoreactive antibody (mu AbGR1) was used as a control.

combined results of all ELISAs revealed quite unequivocally that all antibodies analyzed bound to a surprising variety of antigens in a dose-dependent manner (Figure 3). An overall trend of higher affinity binding toward more complex antigen compositions (extracts of *H felis* and of murine stomach) was noted; no preference for either *Helicobacter*-only reactivity or autoreactivity-only could be detected in any of the antibodies' ELISA profiles. A monoclonal, isotype-matched murine control antibody failed to bind any of the antigens tested (Figure 3). Somatically mutated antibodies did not differ from unmutated antibodies, and evidence of antigen selection in the IgV_H sequence also did not seem to correlate with an increased overall or antigen-specific affinity.

Table 1. VH gene analysis of murine gastric MALT lymphoma cases

Case	VH segment	Homology %	Clones sequenced	Somatic* mutation	FR/CDR	R	S	Antigen selection†	P‡	Intraclonal variation	CDR3 length
mu-1	IGHV1-S130*01	93.06	11	+	FR	42	1	−	.490	+	11
					CDR	29	0	+	.000		
mu-2	IGHV14-3*02	92.36	10	+	FR	13	3	−	.391	+	15
					CDR	6	0	+	.059		
mu-3	IGHV1-S81*02	97.92	13	+	FR	4	2	+	.562	−	13
					CDR	2	0	−	.801		
mu-4	IGHV14-3*02	99.31	10	−	FR	1	1	+	.000	+	13
					CDR	0	0	−	.621		
mu-5	IGHV1-69*02	86.46	8	+	FR	24	6	−	.491	−	10
					CDR	5	4	−	.601		
mu-6	IGHV1-67*1	80.90	10	+	FR	8	5	+	.03	−	12
					CDR	5	2	−	.110		
mu-7	IGHV1-S22*01	94.62	22	+	FR	7	2	−	.112	−	15
					CDR	2	0	−	.009		
mu-8	IGHV14-3*02	98.61	9	−	FR	2	1	+	.329	+	11
					CDR	1	0	−	1.278		
mu-9	IGHV1-18*02	88.19	8	+	FR	18	10	+	.107	+	12
					CDR	5	1	+	.369		
mu-10	IGHV1-S81*02	95.83	10	+	FR	4	1	−	.23	+	14
					CDR	3	3	−	.156		
mu-11	IGHV1-74*01	86.81	10	+	FR	17	9	+	.12	−	12
					CDR	10	2	+	.034		

MALT indicates mucosa-associated lymphoid tissue; FR, framework region; CDR, complementary determining region; R, replacement; S, silent; and mu, murine.

*V_H gene sequences deviating more than 2% from the corresponding germline gene were defined as somatically mutated.

†Presence or absence of positive selection by antigen in the CDR is denoted by + and −, respectively. Presence or absence of negative selection by antigen in the FR is denoted by + and −, respectively, based on the cutoff ratio of 2.9 for replacement to silent mutations.

‡The *P* value was calculated based on the multinomial distribution model and is the probability that excess (for CDR) or scarcity (for FR) of mutations occurred by chance.

Table 2. Immunoglobulin variable heavy and light chain genes of 7 human MALT lymphoma cases

Case	VH segment	Homology %	Clones sequenced	Somatic mutation	Intraclonal variation	JH segment	D segment	CDR3 length	VL segment	JL segment
hu-1	IGHV1-69*01	88.19	8	+	−	IGHJ4*02	IGHD4-17*01	14	IGKV3-20*01	IGKJ4*01
hu-2	IGHV3-7*01	98.61	6	−	−	IGHJ3*02	IGHD3-10*01	17	IGKV1-9*01	IGKJ4*01
hu-3	IGHV3-66*02	93.33	7	+	+	IGHJ4*02	IGHD3-3*01	14	IGKV1-5*03	IGKJ2*01
hu-4	IGHV1-69*01	94.10	6	+	−	IGHJ3*02	IGHD1-1*01	18	IGKV3-20*01	IGKJ4*01
hu-5	IGHV3-33*01	98.61	5	−	+	IGHJ3*02	IGHD5-24*01	12	IGKV1-5*03	IGKJ1*01
hu-6	IGHV1-69*01	96.88	6	+	+	IGHJ5*02	IGHD6-19*01	17	IGKV3-20*01	IGKJ4*01
hu-7	IGHV1-69*05	85.07	8	+	−	IGHJ1*01	IGHD4-23*01	15	IGKV1-9*01	IGKJ5*01

MALT indicates mucosa-associated lymphoid tissue; and hu, human.

Taken together, our results support a diagnosis of polyreactivity. To confirm the specificity of the assay, we performed a series of competitive inhibition ELISAs (Figure 4A-C). To this end, binding of the antibody of interest to one of its targets (immobilized on the ELISA plate) was competitively blocked with increasing concentrations of soluble target of the same or an alternative specificity. In all combinations tested, the soluble-phase antigens were able to competitively block binding to the immobilized target in a dose-dependent manner (Figure 4A-C). As would be expected, high-affinity soluble antigens blocked binding to low-affinity immobilized targets with a higher efficiency than vice versa (Figure 4A-C). BSA, which was used as a negative control, did not block binding even at 100× excess (Figure 4A-B). Overall, the competition results confirmed the specificity of the ELISAs as well as our general observation that complex antigen mixtures constitute higher affinity targets than relatively simple structures such as DNA or LPS.

Sequence analysis of human MALT lymphoma immunoglobulins

To be able to compare the sequence characteristics and specificity of the murine tumor immunoglobulins to their human counterparts, we cloned, sequenced, and recombinantly expressed the antibodies of 7 human low-grade MALT lymphomas.[24] All 7 cases were clonal. Five of the 7 cases showed evidence of somatic hypermutation (Table 2, supplemental Figure 3). Interestingly, we found a bias toward use of the V_H gene segment 1-69*01, which was used in 4 of the 7 cases (Table 2), and has been reported previously for MALT lymphomas of the parotid gland, stomach, and lung.[13] No bias was detected in J and D segment use, but all matching light chains were of the κ isotype (Table 2), another seemingly common bias in MALT lymphoma antibodies.[13,20] Three of 7 cases further showed evidence of intraclonal variation (Table 2, supplemental Figure 4). Interestingly, the CDR3 length of the heavy chains

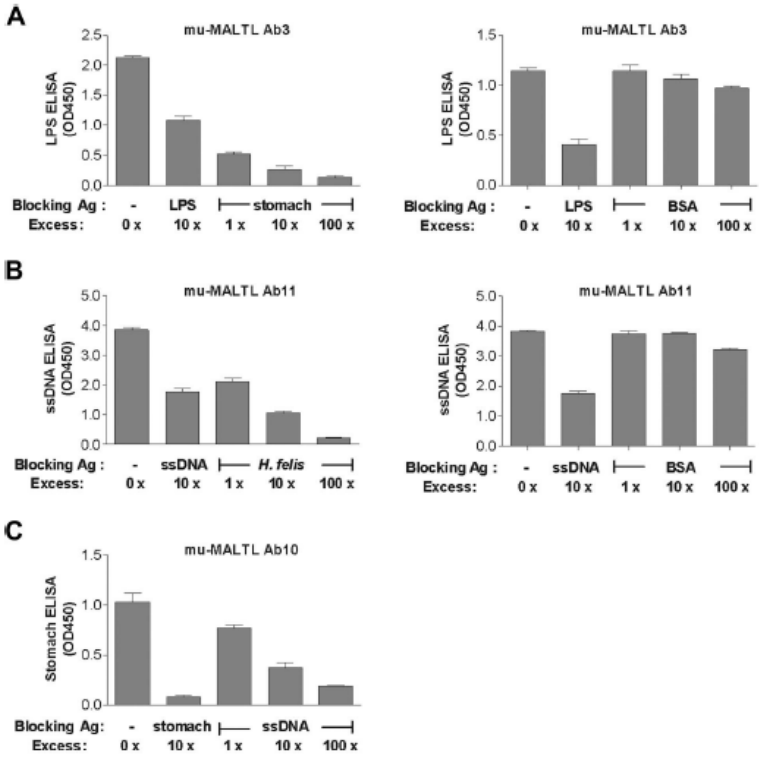

Figure 4. Competitive inhibition ELISAs confirm polyreactive binding patterns of murine MALT lymphoma antibodies. (A-C) A single concentration of the indicated recombinant murine antibodies 3, 11, and 10 (10 μg/mL) was incubated with plate-coated (solid-phase) antigen in the presence of a 10× excess of the same antigen in soluble form or increasing concentrations of an alternative soluble antigen. Three representative inhibition assays are shown and demonstrate the ability of different soluble-phase antigens to inhibit the binding of the antibody to solid-phase antigens. In contrast, BSA did not block binding of Abs 3 and 11 even at 100× excess concentrations (A-B right panels). Error bars represent SEM.

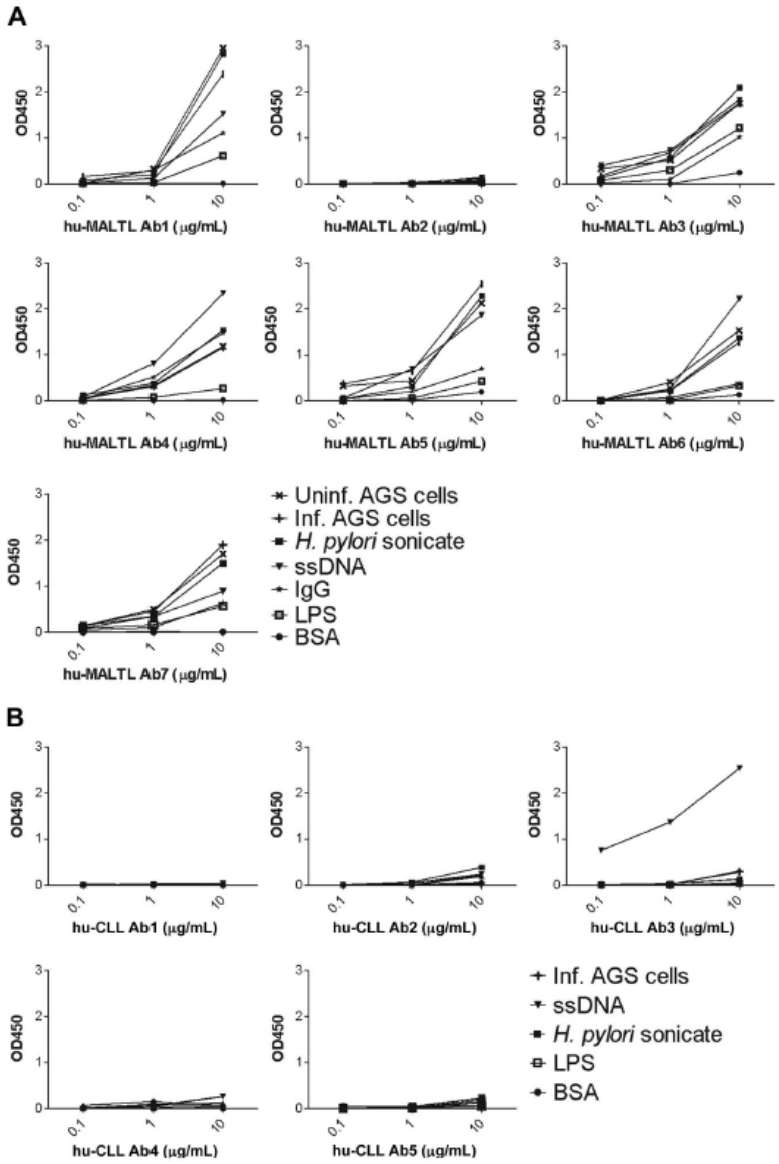

Figure 5. Recombinant human MALT lymphoma–derived antibodies are polyreactive. Three concentrations (0.1, 1, and 10 μg/mL) of 7 recombinant human MALT lymphoma antibodies (A) and 5 recombinant human CLL antibodies (B) were tested by ELISA for reactivity with the antigens indicated in the legends. All MALT lymphoma antibodies, with the exception of Ab2, but none of the CLL antibodies, exhibit a polyreactive binding pattern.

was higher than average (15.3 amino acids as opposed to the 12.7 amino acids usually found in human blood B-cell antibodies),[35] confirming our observation from the murine model. Overall, the sequence analysis of our panel of 7 antibodies confirmed several observations reported previously for human gastric MALT lymphoma and the panel was therefore considered suitable for antigen binding studies.

Antigen reactivity of recombinant human MALT lymphoma–derived antibodies

To determine whether human MALT lymphoma Ig also follows a polyspecific binding pattern, matched heavy and light chains of all 7 human MALT lymphoma cases were screened for reactivity toward BSA, *H pylori* sonicate, LPS, IgG, ssDNA, and AGS cell extract (Figure 5A). All but 1 (hu Ab2) of the human MALT lymphoma antibodies exhibited a polyreactive binding profile very similar to the patterns observed with the murine tumor Igs. Interestingly, hu Ab2 was the only human tumor antibody on the panel to not have undergone either somatic hypermutation or ongoing mutation. As observed for the murine polyreactive MALT lymphoma antibodies, a trend of higher affinity binding toward more complex antigen compositions was evident (Figure 5A). To properly control for the validity of our ELISA readout in the human system, we chose 5 additional non-MALT lymphomas for amplification of their immunoglobulin genes, sequence analysis, and recombinant expression in 293T cells and subsequent ELISA analysis. We selected cases of "mutated" chronic lymphocytic leukemia

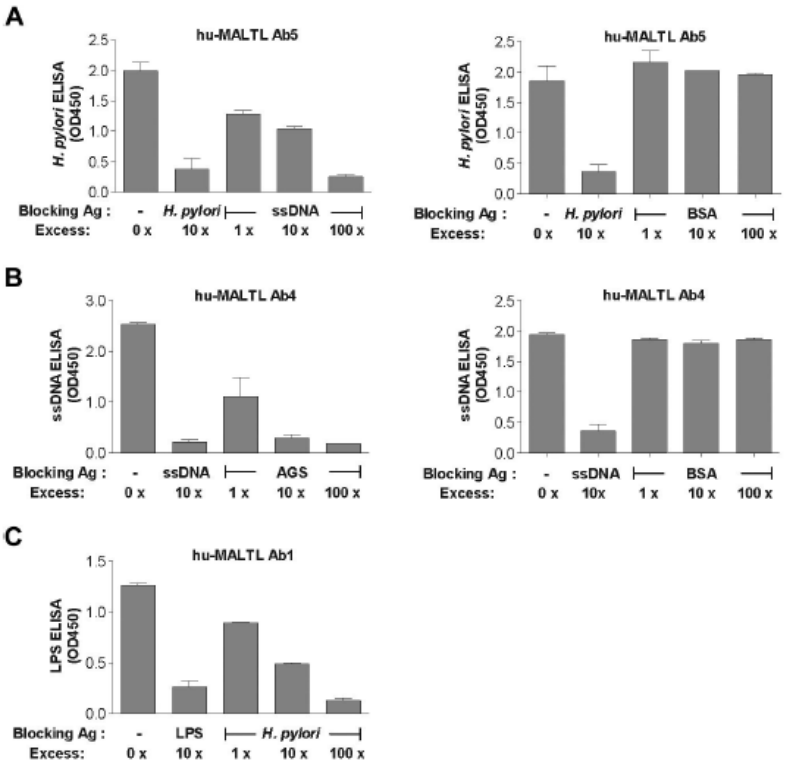

Figure 6. Competitive inhibition ELISAs confirm polyreactive binding patterns of human MALT lymphoma antibodies. (A-C) Three representative competition ELISAs performed as described in the legend to Figure 4 are shown and demonstrate the ability of different soluble targets to inhibit the binding of the recombinant antibody of interest (5, 4, and 1; used at 10 μg/mL) to solid-phase antigens. BSA did not block binding of Abs 5 and 4 even at 100× excess concentrations (A-B right panels). Error bars represent SEM.

(CLL; ie, cases expressing somatically mutated Ig), as these are known to be monoreactive in contrast to their "unmutated" CLL counterparts.[36] All 5 cases were clonal and showed on average 93% homology with the most closely related germline sequence. None of the antibodies displayed a polyreactive binding pattern similar to the one we observed for 6 of the 7 MALT lymphoma Igs. In fact, all but 1 of the 5 CLL antibodies failed to show any affinity for any of the targets of our ELISAs (one—Ab3—bound to ssDNA, but not to any of the other antigens). This result showed that MALT lymphoma antibodies have fundamentally different antigen-binding properties than monoreactive CLL antibodies, and confirms the validity of our ELISA.

Finally, the antigen specificity of the recombinant human MALT lymphoma antibodies was confirmed by a series of competitive inhibition ELISAs (Figure 6A-C); as observed for the murine antibodies, soluble-phase antigens were able to competitively block binding to the immobilized target in a dose-dependent manner, whereas BSA did not block binding even at 100× excess (Figure 6A-C).

To assess the binding affinity of the various tumor Igs, we calculated K_d values for a select group of 6 tumor antibody-antigen complexes using a curve-fitting approach as described.[32] The K_d values ranged from 1.1×10^{-7} M (hu Ab5 reactivity toward LPS) to 6.3×10^{-8} M (hu Ab4 toward *H pylori* extract). Murine tumor antibodies showed a similar range of affinity (eg, $K_d = 2 \times 10^{-8}$ M for mu Ab11 toward ssDNA; $K_d = 5 \times 10^{-8}$ M for mu Ab11 toward stomach extract). Overall, the binding affinity of polyspecific MALT lymphoma–derived antibodies is clearly lower than what is typically observed for monoreactive antibodies (which have K_ds of 10^{-8}-10^{-11} M), but higher than the typical affinity of polyreactive, unmutated IgM expressed by immature B cells (K_ds of 10^{-4}-10^{-7} M).[37]

Discussion

Persistent infection of BALB/c mice with various *Helicobacter* species results in the development of gastric MALT lymphoma in a majority of mice, with "clinical" features that are strikingly similar to the human form of the malignancy: late onset of disease, dependence on active infection with the organisms, and a low propensity for spreading.[10,21-23] We show here that several molecular parameters of the murine tumors also mimic the human disease well: (1) the monoclonal status of more than half of all individually dissected tumors, (2) the antigen dependence of tumor cell proliferation in vitro, (3) the surface exposure of IgM and apparent lack of class switch recombination, (4) the somatic hypermutation of antibody sequences, accompanied in several cases by positive and/or negative antigen selection, and (5) the intraclonal variability that is a hallmark of continuing antigen exposure and, consequently, ongoing somatic mutation. We further demonstrate that both the murine tumor-derived antibodies as well as all but 1 of the antibodies derived from MALT lymphoma patient biopsies show a pattern of polyreactivity, displaying equally strong affinity toward a diverse panel of foreign but also self-antigens. Finally, this result is corroborated by our observation of a strong bias toward the use of V_H gene segments that have previously been associated with autoantibodies or polyreactive antibodies in other B-cell malignancies or autoimmune pathologies.

In the murine setting, we often find that more than 1 tumor arises per stomach, that is, the lymphoma presents as a multifocal disease (as is the case in humans).[38] In those cases in which we were able to dissect multiple monoclonal tumors from the same stomach, they were usually derived from independent clones, confirming that MALT lymphoma is a highly localized disease with little tendency to spread, even within the same organ. The monoclonal status of 55% of individually dissected tumors confirms the validity of our model; clonality rates of human MALT lymphomas range from 63% to 92% of histologically confirmed cases.[20,39] The lower rates of clonality in the murine model may be due to the tiny size and multifocal nature of the murine tumors, which are difficult to dissect without contamination from surrounding normal tissue as well as neighboring tumor material.

Gastric MALT lymphomas are antigen dependent in their early stages, that is, they require the constant presence of as yet unknown antigen(s) for growth in vivo.[9,10,22] Interestingly, this requirement for antigen is retained in ex vivo cultures of single-cell suspensions derived from individual murine tumors, which proliferate only in the presence of *Helicobacter* extract. Despite the apparent complexity of tumor cell suspensions, which contained a significant proportion of admixed T cells and other leukocytes, the proliferating population consisted predominantly of (tumor) B cells.

The immunoglobulin genes of murine as well as human gastric MALT lymphomas are subject to somatic hypermutation but fail to undergo class switch recombination. Indeed, expression of the enzyme responsible for both processes, activation-induced cytidine deaminase, could be detected by reverse-transcription–polymerase chain reaction in material generated from both murine tumor and human MALT lymphomas (data not shown). Although the 2 processes are known to be linked and usually happen simultaneously during germinal center reactions, it is now widely accepted that a fraction of post–germinal center memory cells are IgM^+.[40] In particular, this seeming contradiction has been described multiple times in B-cell malignancies such as Burkitt lymphoma and CLL as well as in extracerebral and central nervous system diffuse large B-cell lymphoma.[41,42] In MALT lymphoma, switch recombination is often "illegitimate," that is, involving only 1 rather than 2 switch sites and leading to an aberrant rearrangement of the IgH switch region not actually accompanied by isotope switching.[42] The fact that the IgV_H genes of both our panels of murine and human tumor antibodies are somatically hypermutated and, at least in approximately 50% of cases, are subject to ongoing mutation suggests that antigen plays a role during the initiation and the progression of the tumor.

To examine the reactivity of 5 murine and 7 human tumor immunoglobulins with various autoantigens and foreign antigens, we recombinantly expressed them as murine and human IgG, respectively; this approach ensured that the specificity of the recombinant antibodies indeed corresponded with that of the tumor while maintaining sufficiently high avidity. Previous efforts, with the exception of one report,[20] have identified a multitude of alternative targets with the shared characteristic of being autoantigens.[18,19,43] For example, Bende et al found that 7 of 10 gastric and nongastric MALT lymphoma antibodies had rheumatoid factor activity, that is, they bound to auto-IgG.[13]

Our study confirms binding of MALT lymphoma antibodies to IgG. However, with roughly the same or an even higher affinity, our panel of antibodies also bound to a variety of other targets, including *Helicobacter*-derived antigens and stomach extract. The reactivity was nevertheless "specific" in the sense that binding could be blocked to a solid-phase target by an excess of an alternative, soluble target in a concentration-dependent manner. The prevalence of polyreactivity in our panel of antibodies is clearly higher than that of normal peripheral mature B cells, in which the polyreactivity rate is estimated to be less than 5%.[44]

Polyreactivity is not a new phenomenon in the context of B-cell malignancies. It has been described in detail for CLL, which, like MALT lymphoma, is believed to be an antigen-driven malignancy. A majority of unmutated, but not of mutated, CLL-derived antibodies tested with respect to their specificity have revealed polyreactive patterns in previous studies, binding to DNA, IgG, insulin, and LPS.[36,45] In contrast, mutated CLL-derived antibodies are typically monoreactive.[36,45] Indeed, none of the mutated 5 CLL antibodies we included as controls in our analysis showed evidence of polyreactivity. With respect to the link between mutational status and antigen reactivity, CLL-derived antibodies thus differ clearly from MALT lymphoma–derived antibodies. In fact, we found that the only human MALT lymphoma Ig that failed to exhibit polyreactivity was also the only unmutated antibody among those analyzed. It will be interesting to see whether the link between mutation status and polyreactive binding profile holds true in larger studies that include more of the rare unmutated cases.

Both polyreactive CLL[46] and the MALT tumor immunoglobulins reported here share a strongly biased use of the IgV_H1-69 gene segment; 4 of 7 of our panel of human MALT lymphoma antibodies use this segment and, of these, 3 combine the IgV_H1-69 heavy chain with the same light chain V_L segment IGKV3-20*01. This exact combination is typical of rheumatoid factors, with IgV_H1-69 being preferentially used in autoimmune gammopathies reactive toward human IgG such as cryoglobulinemia and Waldenstrom macroglobulinemia.[47,48] IgV_H1-69 was also found to be used in a biased fashion in nongastric MALT lymphomas, including those of the parotid gland, tonsil, and lung.[13] Overall, the strongly biased use of IgV_H1-69 in MALT lymphoma and CLL is consistent with their shared polyreactive antibody specificity, as well as their shared dependence on antigenic stimulation.

Polyreactivity is thought to be caused by a special flexibility of the antigen-binding pocket, with multiple configurations or "isomers" of an antibody existing even before exposure to antigen[49,50]; some evidence suggests that a longer CDR3 region of polyreactive antibodies might be underlying this flexibility.[51] Indeed, we find that our human as well as our mouse MALT lymphoma antibodies possess longer than average CDR3 regions. In conclusion, many of the known molecular and structural hallmarks of polyreactive antibodies such as IgM class, CDR3 length, and preferential V_H gene use are detected in MALT lymphoma antibodies and are consistent with our experimental finding of polyreactivity, suggesting a role for antigenic stimulation in MALT lymphoma pathogenesis.

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Authorship

Contribution: V.J.C. designed and performed research, analyzed data, and wrote the paper; I.A. and C.G. collected experimental animal material and performed fluorescence-activated cell sorting analyses; M.Q.H., T.W., A.N., and C.R. collected patient material and extracted mRNA; S.F. contributed vital tools and helped analyze data; and A.M. designed research, performed research, analyzed data, and wrote the paper.

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3.2 B-cell receptor signalling and CD40 ligand-independent T cell help cooperate in *Helicobacter*-induced MALT lymphomagenesis

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Summary: In order to assess the functional consequences of the polyreactive nature of MALT lymphoma B cells, transient *ex vivo* cultures of murine MALT lymphoma cells were established. Explanted tumour B-cells were found to proliferate upon stimulation with the same panel of self and foreign antigens that are recognised by their surface antibodies. We next set out to clarify the role of infiltrating T cells in the MALT lymphoma environment. The depletion of $CD4^+$ and $CD25^+$ T cells prevented the proliferation of tumour cells both *ex vivo* and *in vivo*. This pro-tumourgenic influence was independent from direct contact between the T and B cells via CD40/CD40L signalling. A significant proportion of tumour-infiltrating $CD4^+$ T cells were found to be highly suppressive $CD25^+FoxP3^+$ regulatory T cells (Treg). These cells were actively recruited by the tumour cells through secretion of the Treg-attracting chemokines CCL17 and CCL22. In summary, our data suggest that B-cell receptor-derived signals cooperate with T-helper cell signals in driving the progression of MALT lymphoma, providing an explanation for the unique antigen dependence of this B-cell malignancy.

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ORIGINAL ARTICLE

B-cell receptor signaling and CD40 ligand-independent T cell help cooperate in *Helicobacter*-induced MALT lymphomagenesis

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Gastric B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) develops in the context of chronic inflammation caused by *Helicobacter pylori* infection. Most pathophysiological features of the early stages of MALT lymphomagenesis can be reproduced by experimental infection of BALB/c mice with *Helicobacter* species. We have previously shown that MALT lymphomas are infiltrated by T-helper cell type 2-polarized T cells and that human and murine tumor B cells carry polyreactive surface immunoglobulins. Using the murine model of the disease, in this study we show that explanted tumor B cells proliferate upon stimulation with the same panel of self and foreign antigens that are recognized by their surface antibodies. Tumor cell proliferation is strongly enhanced by the presence of intratumoral $CD4^+$ T cells in a CD40/CD40L-independent manner. A large proportion of tumor-infiltrating $CD4^+$ T cells are $CD25^+FoxP3^+$ regulatory T cells (Tregs) with highly suppressive activity, which are recruited by the tumor cells through secretion of the Treg-attracting chemokines CCL17 and CCL22. The depletion of $CD25^+$ cells was as efficient as $CD4^+$ T cell depletion in blocking tumor growth *in vitro* and *in vivo*. In conclusion, our data suggest that B-cell receptor-derived signals cooperate with T-helper cell signals in driving the progression of MALT lymphoma, providing an explanation for the unique antigen dependence of this B-cell malignancy.
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Introduction

Extranodal marginal-zone B-cell lymphomas of mucosa-associated lymphoid tissue (MALT) comprise a group of low-grade non-Hodgkin's lymphomas that arise in the context of chronic lymphoid proliferation in a variety of mucosal sites. A majority of cases are found in the stomach, in which they develop against a background of chronic active gastritis caused by the Gram-negative bacterium *Helicobacter pylori*.[1,2] In a subset of infected individuals, *H. pylori*-induced chronic inflammation can give rise to organized lymphoid tissue, from which individual malignant clones grow out and form the lymphoepithelial lesions typical of gastric MALT lymphoma.[2] Low-grade MALT lymphomas are characterized by an indolent clinical course; they grow slowly, rarely undergo high-grade transformation and typically remain localized to their primary site.

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In its early stages, MALT lymphoma is believed to be an antigen-dependent disease, relying on antigenic and/or helper T cell-derived growth signals and being incapable of autonomous growth.[1,3,4] Several lines of evidence argue that, in the case of gastric MALT lymphomas, the antigenic signals are provided by infection with *H. pylori*. Ongoing or past infection with the bacterium is detectable in a large majority of cases,[1,2,5] and eradication of the bacterium leads to regression of early-stage tumors.[3,4] In certain mouse strains, long-term experimental infection with *H. pylori* or its close relatives *H. felis* or *H. heilmannii* is sufficient to induce gastric B-cell lymphomas that histologically and biologically resemble the human disease counterpart.[6–8] The murine tumors are slow growing, regress upon eradication of the infection and recur rapidly upon re-introduction of the bacterium.[6,7] Despite its generally benign nature, a small fraction of gastric MALT lymphomas ultimately undergo high-grade transformation or acquire one of several known characteristic chromosomal translocations,[9] thereby rendering the lymphoma antigen independent and refractory to *H. pylori* eradication therapy. The shared outcome of all chromosomal translocations identified to date is the constitutive activation of the nuclear factor-κB signaling pathway, leading to B-cell receptor (BCR)-independent proliferation of the tumor B cells.[9] We and others have reported previously that gastric MALT lymphomas are infiltrated by large numbers of T-helper cells expressing interleukin-4 and other T-helper cell type 2 cytokines.[7,10] The tumor B cells carry rearranged somatically mutated immunoglobulins on their surface,[11–14] which have undergone positive selection.[13,15] We have recently shown that recombinant tumor immunoglobulins derived from both human and murine MALT lymphomas are polyreactive; that is, they bind to a panel of foreign and autoantigens with similar affinity.[14] Moreover, the tumor cells preferentially use the Ig heavy chain V_H gene segment 1-69 that has previously been linked to polyreactive and autoantibodies in other B-cell malignancies or autoimmune pathologies.[14]

Using the BALB/c mouse model of *Helicobacter*-induced gastric MALT lymphoma, in this study we elucidate the relative contribution of BCR-derived and T-helper cell-derived signals in MALT lymphoma growth *in vitro* and *in vivo*. We observe that purified tumor B cells proliferate in response to the same range of antigens that are recognized by recombinant tumor Ig. Tumor-infiltrating T cells contribute to maximal tumor cell proliferation. A significant fraction of tumor-infiltrating T cells are $CD25^+$ regulatory T cells (Tregs) with strong suppressive activity, which are recruited by the tumor B cells in a CCL17/CCL22-dependent manner. The depletion of either Tregs or all $CD4^+$ T cells *in vivo* efficiently induces tumor regression, implying that T cell-derived signals are required for MALT lymphomagenesis.

Materials and methods

Animal experimentation, tumor cell cultures and proliferation assays

Specific pathogen-free female BALB/c mice were infected orally at 6 weeks of age with three consecutive doses of $\sim 5 \times 10^7$ *H. felis* (CS1, American Type Culture Collection 49179) and maintained for 18 months in individually ventilated cages. For *in vivo* depletion of T cells, mice received biweekly intraperitoneal injections of 300 µg anti-CD4 monoclonal antibody (YTS 191; Bio X Cell, West Lebanon, NH, USA) or anti-CD25 monoclonal antibody (PC-61.5.3; Bio X Cell). All animal experiments were performed in accordance with the institutional policies and were approved by the cantonal veterinary office. Upon killing by CO_2 inhalation, the stomachs were removed and dissected along the lesser curvature; longitudinal sections of each stomach were fixed in 10% neutral-buffered formalin before paraffin embedding, sectioning and hematoxylin and eosin staining. Another section was subjected to isolation of genomic DNA followed by quantitative analysis of *Helicobacter* colonization using real-time PCR as described.[16] From the remaining stomach tissue, tumors of >1 mm diameter were excised, and single-cell suspensions were generated and cultured in RPMI supplemented with 10% fetal calf serum and penicillin/streptomycin. *Helicobacter* lysate, lipopolysaccharide, single-stranded DNA, immunoglobulin G (IgG), gastric mucosal protein extract and bovine serum albumin were added at 10 µg/ml. *Helicobacter* lysate and gastric mucosal extract were prepared as described previously.[14] Supernatants of tumor cell suspensions were harvested for Treg conversion assays as described in the Supplementary Methods. $CD4^+$, $CD25^+$ and $CD154^+$ T cells were depleted from the tumor cell suspensions by immunomagnetic depletion using biotinylated monoclonal antibodies (anti-CD4 clone RM4-5, anti-CD154 clone MR1, both from BD Biosciences, CA, USA; anti-CD25 clone PC61.5, R&D Systems, Minneapolis, MN, USA) followed by streptavidin-coated magnetic beads. Blocking of the CD40/CD40L interaction was achieved with 10 µg/ml of anti-CD40L antibody (clone MR1, low endotoxin, azide free; BD Biosciences). Tumor cell proliferation was quantified by $[^3H]$-thymidine incorporation assay, or by 5-bromodeoxyuridine (BrdU) incorporation, followed by fluorescence-activated cell sorting analysis using a fluorescent *in situ* cell proliferation kit (Roche Diagnostics, Mannheim, Germany) in combination with a fluorescently labeled anti-CD19 antibody (clone 6D5; Abcam, Cambridge, UK). Tumor cell suspensions were phenotyped by fluorescence-activated cell sorting and subjected to immunomagnetic isolation of Tregs as described in the Supplementary Methods. Tumor-derived Tregs were assessed for their ability to migrate and to suppress effector T-cell proliferation, as described in detail in the Supplementary Methods.

Patient material

Human patient material was obtained from seven patients with gastric low-grade MALT lymphoma who were part of a previously published study conducted at Philipps University Hospital (Marburg, Germany).[17] All tumors were diagnosed as *H. pylori*-positive low-grade gastric MALT lymphomas and all were negative for the translocation t(11;18)(q21;q21). A tumor biopsy as well as biopsy material from adjacent unaffected tissue (with histological evidence of chronic gastritis) was secured from all seven patients. For the histopathological analysis of archived patient material, consecutive cases of *H. pylori*-positive gastritis, of *H. pylori*-positive gastric low-grade MALT lymphoma and of gastric high-grade MALT lymphoma were drawn from the surgical pathology files of the Institute of Pathology at the Cantonal Hospital (St Gallen, Switzerland) and the Institute of Pathology, Klinikum Bayreuth, (Bayreuth, Germany). All data were blinded to guarantee patients' protection. This procedure is in agreement with the guidelines for use of human material in research issued by the ethics committees of both institutions. The patient material was used for the quantitative analysis of FoxP3 and CD4 expression by immunohistochemistry and real-time reverse transcriptase-PCR, as described in detail in the Supplementary Methods.

Results

Murine MALT lymphoma B cells proliferate ex vivo *in response to antigens recognized by their BCR*

We have reported recently that recombinantly expressed antibodies consisting of matching heavy and light chain sequences derived from murine and human MALT lymphomas bind to a variety of target antigens, including autoantigens such as IgG and single-stranded DNA, as well as foreign antigens such as *Helicobacter* and *Escherichia coli* extract or lipopolysaccharide.[14] To investigate the functional consequences of antigen binding by the tumor cells, which are IgM^+ and express functional BCR on their surface,[14] we generated single-cell suspensions from macroscopically discernible tumors that we dissected from the stomachs of *H. felis*-infected BALB/c mice. The tumor cell suspensions were cultured for 5 days in the presence of *Helicobacter* extract, lipopolysaccharide, gastric mucosal extract, IgG and single-stranded DNA before quantification of proliferation by $[^3H]$-thymidine incorporation. The whole panel of cognate antigens stimulated tumor cell proliferation compared with untreated controls (Figure 1a), whereas bovine serum albumin, which is not recognized by recombinant tumor Ig,[14] did not induce proliferation (Figure 1a). In parallel to culturing unsorted tumor suspensions, which contained a significant portion of admixed non-B cells (see below), we specifically enriched for the tumor B cells by immunomagnetic depletion of all other cell types. The resulting B-cell populations (which were >95% pure) showed a similar relative reactivity toward the panel of target antigens (Figure 1b); however, their overall proliferative response was reduced by 90% compared with the unsorted cultures. The results confirm that MALT lymphoma B cells carry functional, polyreactive Ig on their surface and may receive proliferative signals through their BCR. However, additional tumor-infiltrating cell types are required for a maximal response to cognate antigen.

Murine MALT lymphomas are infiltrated by activated, immunocompetent $CD4^+$ T cells

To quantify and phenotype the tumor-infiltrating cell populations, we generated single-cell suspensions from 10 independent tumors and analyzed their expression of T cells, B cells and myeloid markers. As shown in previous studies, a majority of leukocytes in the suspensions were $CD19^+$ B cells (on average 67%, Figure 2a); a small fraction was positive for the myeloid markers CD11b or CD11c (2%, Figure 2a). Most remaining cells stained positive for CD3 (on average 19.2%, Figures 2a and b). A majority of $CD3^+$ T cells were $CD4^+$ (83.2%, Figures 2a and b), some of which expressed the activation marker CD69 (29.5%, Figure 2b). $CD69^+$ activated T cells expressed significantly higher levels of the B-cell co-stimulatory molecule CD40L than the resting population (Figure 2b), suggesting that

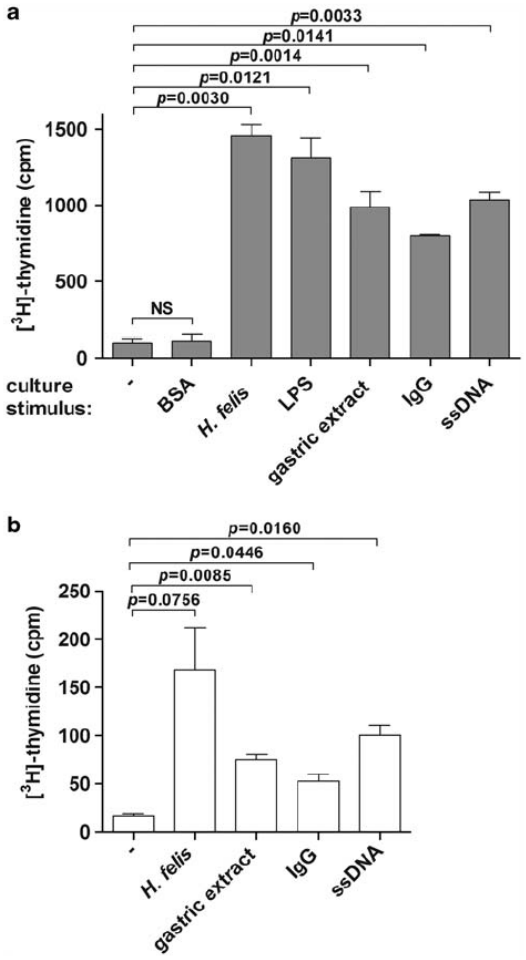

Figure 1 MALT lymphoma B cells proliferate *in vitro* in response to known target antigens of their B-cell receptor. Five tumors, >1 mm in diameter, were excised from the stomachs of two BALB/c mice that had been infected with *H. felis* for 18 months. Single-cell suspensions were prepared and were either seeded directly into a round-bottomed 96-well plate at a density of 7.5×10^4 cells per well (**a**) or subjected to immunomagnetic isolation of the tumor B-cell population. Pure B cells (>95%) were seeded at 2.5×10^4 cells per well (**b**). Both populations were treated with the indicated reagents at 10 μg/ml final concentration. Proliferation was determined by [^{3}H]-thymidine incorporation after 5 days in culture. Averages and s.d. are shown for two replicate wells per condition. Data are representative of three independent experiments.

tumor-infiltrating T cells are immunocompetent. Both subsets were positive for CD28 (data not shown).

T-helper subsets are required for efficient induction of tumor B-cell proliferation in vitro

To elucidate a possible functional role for tumor-infiltrating T cells in MALT lymphoma cell proliferation, tumor cell suspensions were depleted immunomagnetically of their CD4$^+$, CD40L$^+$ and CD25$^+$ T-cell subsets. The depleted cultures were compared with the corresponding undepleted cell suspension with respect to *Helicobacter* extract-induced proliferation as determined by [^{3}H]-thymidine incorporation (Figure 3a). The depletion of all CD4$^+$ cells or of CD40L$^+$ cells significantly reduced the overall proliferation; the depletion of CD25$^+$ T cells had a weaker, but nevertheless significant effect (Figure 3a). To confirm that the observed reduction in overall proliferation was indeed because of a reduction of tumor B-cell proliferation rather than the loss of (proliferating) T cells, we subjected parallel cultures to BrdU incorporation followed by the fluorescence-activated cell sorting-based quantification of BrdU$^+$ cells (Figure 3b). Indeed, the majority of BrdU$^+$ cells were CD19$^+$ in the undepleted culture, and the numbers of BrdU$^+$ CD19$^+$ cells were reduced in the depleted cultures (Figure 3b, upper and lower panels). The remaining CD19$^-$ BrdU$^+$ fraction stained positive for CD4, suggesting that tumor-infiltrating T cells also proliferate under these conditions (data not shown). Interestingly, despite the strong effect of CD40L$^+$ T-cell depletion on tumor B-cell proliferation, inhibition of the B-/T-cell interaction through blocking of CD40L with an antagonistic antibody did not abrogate proliferation (Figure 3a). The effectiveness of the CD40L blocking antibody at this concentration was confirmed in co-cultures of purified T cells and B cells using cytokine expression as a functional read-out of the direct interaction between both cell types (data not shown). The combined results suggest that activated CD40L$^+$ T cells are essential for providing helper signals, but these are independent of a direct interaction between CD40 and its ligand.

In a complementary approach designed to assess the role of T cells in tumor B-cell proliferation, we supplemented undepleted tumor cell suspension cultures (Figure 3c, in gray) and purified tumor B-cell suspensions (Figure 3c, in white) with autologous conventional (CD4$^+$CD25$^-$) or regulatory T cells (CD4$^+$CD25$^+$), which were isolated from the spleens of the tumor donors. Addition of both T-cell populations significantly enhanced proliferation upon *Helicobacter* treatment of the undepleted tumor cell suspension (and had similar, although weaker, effects on the pure B-cell cultures, Figure 3c), suggesting that T cells can provide stimulatory signals to tumor B cells even if they do not originate from the tumor itself.

Intratumoral Tregs are highly suppressive in vitro

On the basis of the effect that the depletion of intratumoral CD25$^+$ cells has on tumor cell proliferation, we hypothesized that the tumors should be enriched for Tregs. We confirmed this by quantitative expression analysis of the Treg lineage-defining transcription factor FoxP3 (Figure 4a); dissected murine gastric MALT lymphomas expressed significantly higher levels of FoxP3 than directly adjacent tissue with histologically confirmed gastritis and also more than the gastric mucosa of an uninfected age-matched mouse (Figure 4a). To assess the suppressive activity of intratumoral Tregs in a standardized suppression assay, we immunomagnetically isolated CD4$^+$CD25$^+$ cells from tumor cell suspensions and from the corresponding mesenteric lymph nodes and spleen. Treg yields were between 6 and 8.3% of the total tumor cell population. The CD25$^+$ Treg populations were mixed with effector T cells in a 1:1 ratio, and the co-cultures were stimulated with anti-CD3/CD28 monoclonal antibody-coated beads (Figure 4b). The suppressive activity of intratumoral Tregs was significantly higher than that of the splenic and mesenteric lymph node-derived Tregs isolated from the same donor (Figure 4b). We conclude from these results that the tumors are infiltrated by Tregs that are highly

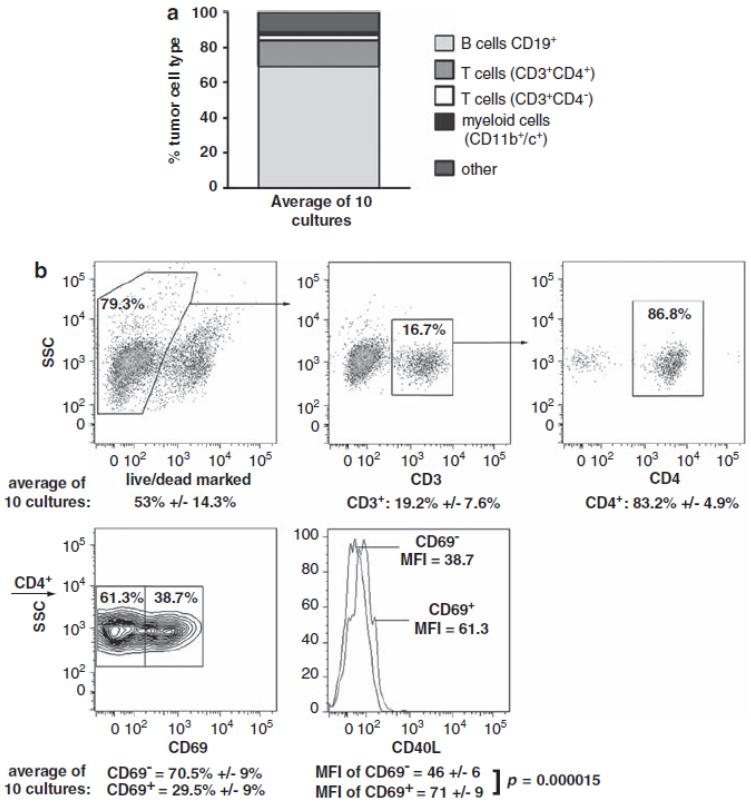

Figure 2 MALT lymphoma-infiltrating T cells are predominantly $CD4^+$ and express CD69 and CD40L. (**a**, **b**) Ten tumors, >1 mm in diameter, were excised from the stomachs of five BALB/c mice that had been infected with *H. felis* for 18 months. Single-cell suspensions were prepared, stained for surface expression of CD19, CD11b, CD11c, CD3, CD4, CD69 and CD40L and analyzed by flow cytometry. (**a**) The average tumor composition is shown for all 10 tumor cultures. (**b**) Fluorescence-activated cell sorting (FACS) plots are shown for the T cell compartment of a representative tumor; the averages ± s.d. of all 10 tumors are indicated below the dot plots and histogram. MFI, median fluorescence intensity.

suppressive toward effector T cells on one hand, and essential for optimal tumor B-cell proliferation on the other.

Tregs are recruited to MALT lymphomas through the activity of tumor B cell-derived chemokines

Tregs are actively recruited by a variety of epithelial cancers, and this phenomenon is believed to benefit the tumors by blocking local antitumor immune responses.[18–20] Treg recruitment into the microenvironment of ovarian and gastric cancer requires the activity of either or both of the two chemokines, CCL17 and CCL22,[18,21] which bind to a shared receptor on the Treg surface, CCR4. To investigate whether MALT lymphoma B cells might actively recruit Tregs to the tumor mass using the same mechanism, we first investigated CCL17 and CCL22 expression in tumor compared with matched gastritic tissue and uninfected normal gastric mucosa (Figure 5a). Indeed, transcript levels of both chemokines were significantly higher in the tumors compared with adjacent inflamed tissue or normal mucosa (Figure 5a).

To measure the chemoattraction of spleen-derived natural ($CD4^+CD25^+$) Tregs by MALT lymphoma supernatants generated by culturing single-cell suspensions *ex vivo* as described above (Figures 1 and 3), we established a transwell migration assay in which the Tregs were fluorescently labeled and allowed to migrate toward supernatants of tumor cell cultures. The supernatants of cultures that had been induced to proliferate by addition of *Helicobacter* extract recruited Tregs more efficiently than the supernatants of unstimulated tumor cells (Figure 5b). The depletion of total $CD4^+$, $CD40L^+$ or $CD25^+$ T cells, which prevented tumor cell proliferation upon stimulation (Figure 3), also significantly reduced the chemoattraction of Tregs by the corresponding supernatants (Figure 5b). Purified tumor B cells that had been stimulated with *Helicobacter* extract also induced less migration than the undepleted culture (Figure 5b). Interestingly, Treg migration could be partially blocked by neutralization of either CCL17 or CCL22. Neutralization of both chemokines together completely abrogated Treg migration toward the supernatants of all cultures (Figure 5b). In summary, the results imply that actively growing (but not resting) tumor cells possess the ability to recruit Tregs; this process is mediated by two partially redundant chemokines that bind to the same receptor, CCR4. The observation that Treg chemoattraction is maintained at least partially in pure B-cell cultures and is

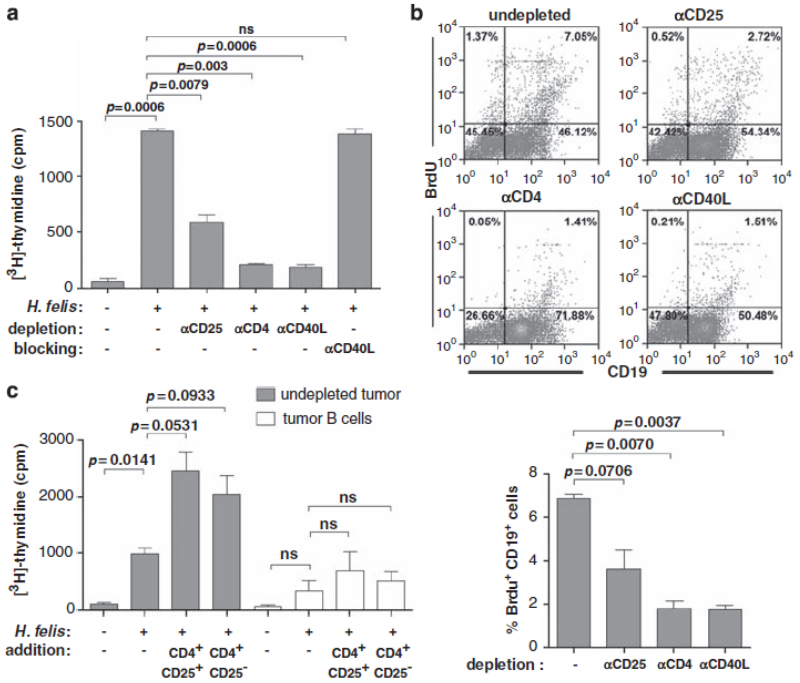

Figure 3 Tumor B-cell proliferation upon *Helicobacter felis* stimulation requires soluble T-helper cell-derived signals. (**a**, **b**) Single-cell suspensions were generated from five tumors and depleted of $CD25^+$, $CD4^+$ or $CD40L^+$ T cells by immunomagnetic depletion or were left untreated. Where indicated, a blocking antibody to CD40L was added at 10 μg/ml. Proliferation upon stimulation with *H. felis* extract was determined by [^{3}H]-thymidine incorporation (**a**) and BrdU incorporation (**b**) after 5 days in culture. $BrdU^+CD19^+$ cells and $BrdU^+CD19^-$ cells were quantified flow cytometrically; representative dot plots are shown in the upper panel, and averages of at least two and up to three replicate wells are shown in the lower panel. (**c**) Single-cell suspensions were generated from 10 tumors isolated from a single mouse (in gray); half of the preparation was further subjected to immunomagnetic isolation of pure tumor B cells (in white). Both types of cultures were further supplemented with $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells isolated from the spleen of the donor mouse. Most cultures were stimulated with *H. felis* extract as indicated. Averages of two to three replicates per condition are shown. Data are representative of two independent experiments.

approximately proportional to their level of proliferation suggests that the tumor B cells themselves rather than tumor-infiltrating T cells are the source of these chemokines.

Soluble, tumor-derived signals convert naive $CD4^+$ T cells to $FoxP3^+$ Tregs

It is now well established that $FoxP3^+$ Tregs not only arise in a thymus-dependent process (the 'natural' or nTreg subset), but can also be generated in the periphery through a combination of T-cell receptor-mediated signals and transforming growth factor-β (TGF-β) exposure (the so-called 'inducible' Treg). To explore whether Tregs can be converted from conventional T cells directly in the tumor microenvironment in addition to being recruited there, we cultured reporter cells expressing enhanced green fluorescent protein under the *FoxP3* promoter in the presence of tumor cell culture supernatant (Figure 6a). Whereas the supernatants of unstimulated tumor cell cultures had no effect on FoxP3 expression, supernatants of *Helicobacter* extract-stimulated (that is, proliferating) tumor cells induced the conversion of conventional T cells to $FoxP3^+$ Tregs almost as efficiently as a positive control consisting of a CD3-crosslinking antibody and recombinant interleukin-2 and TGF-β (Figure 6a). Supernatants from parallel cultures of the corresponding splenocytes from the same donor did not induce Treg conversion, independent of whether the splenocytes had been stimulated by *Helicobacter* extract or not. These data were confirmed by quantitative real-time reverse transcriptase–PCR analysis of FoxP3 expression in a parallel experiment (Figure 6b). Overall, the data suggest that proliferating MALT lymphoma cells produce as-yet-unidentified soluble factors that allow them to convert Tregs from conventional T cells *in situ* in addition to recruiting them through the activity of Treg chemoattractants.

Depletion of $CD4^+$ or $CD25^+$ cells in vivo *induces MALT lymphoma regression*

To determine whether $CD4^+$ T cells and their $CD25^+$ subset would be as essential for tumor cell proliferation *in vivo* as they are *in vitro* (Figure 3), we depleted both subsets with neutralizing antibodies during the last 3 months of an 18-month infection experiment. *H. felis* infection-induced gastric MALT lymphomas start to appear as macroscopically visible nodules between 15 and 18 months after infection,[6,8] leaving a therapeutic window of 3 months before the mice become morbid as a result of their lymphoma burden. Upon killing, the stomachs were removed and macroscopically visible tumors were counted (Figure 7a). The infected mice that had not received T-cell-depleting antibodies had developed anywhere

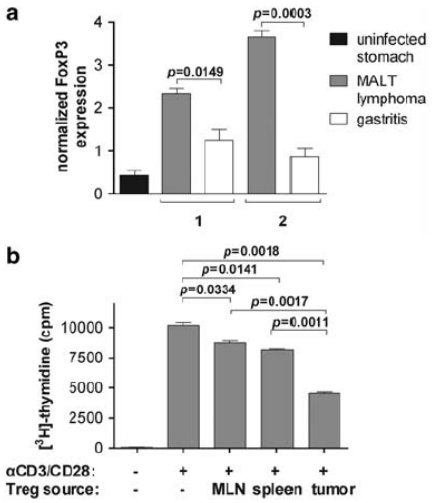

Figure 4 Intratumoral $CD4^+CD25^+$ T cells have suppressive activity *in vitro*. (**a**) To assess whether murine MALT lymphomas are enriched for Tregs, FoxP3 expression was quantified by real-time reverse transcriptase-PCR (RT-PCR) using RNA from two tumors (derived from two mice) and corresponding adjacent gastritis tissue as well as mucosa from an uninfected stomach. FoxP3 expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Averages and s.d. of three replicates are shown. (**b**) To assess whether intratumoral Tregs possess suppressive activity *in vitro*, $CD4^+CD25^+$ T cells were immunomagnetically isolated from a tumor cell suspension generated from five pooled tumors. $CD4^+CD25^-$ T cells isolated from the corresponding spleen were stimulated with anti-CD3/CD28-coated Dynabeads for 5 days, with or without addition of an equal number of $CD4^+CD25^+$ Tregs derived either from the tumor or the spleen or the mesenteric lymph nodes (MLNs) of the donor mouse. Proliferation was determined by $[^3H]$-thymidine incorporation. Averages of triplicates are shown. Data are representative of two independent experiments.

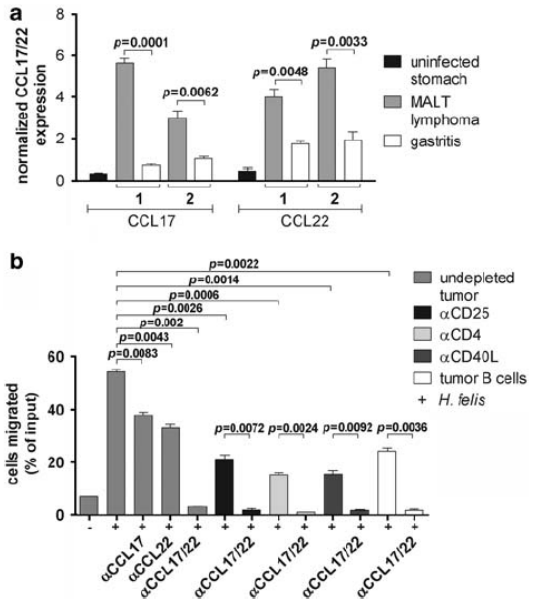

Figure 5 Treg migration is induced by MALT lymphoma B cells in a CCL17- and CCL22-dependent manner. (**a**) Expression of the Treg-recruiting chemokines CCL17 and CCL22 was assessed in the same samples as shown in Figure 4. Transcript levels of the chemokines were quantified by real-time reverse transcriptase-PCR (RT-PCR) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. (**b**) To assess Treg chemoattraction by the supernatants of tumor cell cultures, $CD4^+CD25^+$ Tregs were isolated from the spleen of an uninfected donor mouse, labeled with the acetomethoxy derivate of calcein (calcein AM) and seeded into the upper chamber of a transwell chemotaxis plate. Supernatants collected from the undepleted tumor cell cultures (stimulated or unstimulated, indicated by − and +) as well as from the CD4-, CD40L- and CD25-depleted cultures shown in Figure 3 were placed in the lower chamber. The supernatants collected from purified tumor B cells (Figure 1b) were tested as well. Where indicated, neutralizing antibodies to CCL17 and/or CCL22 were added to the lower chamber at 2 μg/ml. Treg migration was quantified fluorospectrometrically; the fraction of migrated cells in percentage of input was calculated based on a standard curve of known cell number. Averages and s.d. of duplicate readings are shown. The effect of CCL17 and CCL22 neutralization was confirmed four times on undepleted supernatants and twice on depleted supernatants.

from 2 to 15 tumors, predominantly at the forestomach/corpus junction along the greater curvature. One mouse was still tumor free at 18 months (Figure 7a). In contrast, none of the $CD4^+$ T-cell-depleted mice had developed lymphoma and only one mouse of the $CD25^+$ T-cell-depleted group had a single tumor (Figure 7a). The depletion efficiency with the CD4-depleting antibody at the time of killing was on average 99.6%, as determined flow cytometrically in the spleens of all mice of the group, whereas the depletion of $CD25^+$ cells was on average 89.3% efficient for the $CD4^+CD25^+$ subset of T cells, and reduced the total subpopulation of splenic $CD4^+$ T cells by 28.3%. Histopathological analysis of the three groups of mice confirmed the macroscopical observations (Figure 7b). Only mild-to-moderate symptoms of gastritis could be detected in the mice of the depleted groups, whereas the untreated groups harbored multiple histologically evident tumors (Figure 7b). *Helicobacter* colonization was reduced in the CD4-depleted group but not the CD25-depleted group (Figure 7c) compared with the untreated mice, possibly because of the fact that the mucosal lining of the CD4-depleted mice showed histological signs of atrophy (Figure 7b), a condition that does not support growth of the bacteria.[16] In conclusion, the depletion of $CD4^+$ and $CD25^+$ T cells *in vivo* efficiently blocked lymphomagenesis or led to the regression of pre-existing tumors.

Human low-grade gastric MALT lymphomas are heavily infiltrated by FoxP3$^+$ Tregs and express the Treg-recruiting chemokines CCL17 and CCL22

To investigate whether human gastric MALT lymphomas are as heavily infiltrated by $CD4^+$ T cells and Tregs as their murine disease counterparts, we obtained archived material from patients with low-grade ($n=10$) or high-grade MALT lymphoma ($n=7$; Figures 8a and b). Several cases with gastritis were assessed for comparison ($n=4$). Consecutive paraffin sections were immunohistochemically stained for CD4 and FoxP3; hematoxylin and eosin-stained sections were used to confirm the initial diagnosis (Figure 8a). We quantified the $CD4^+$ and $FoxP3^+$ populations infiltrating the tissues by counting a minimum of 500 cells per specimen (Figure 8b). Whereas in the low-grade lymphomas an average of 15.6% of cells in the tumors were $CD4^+$, this number was lower in the high-grade

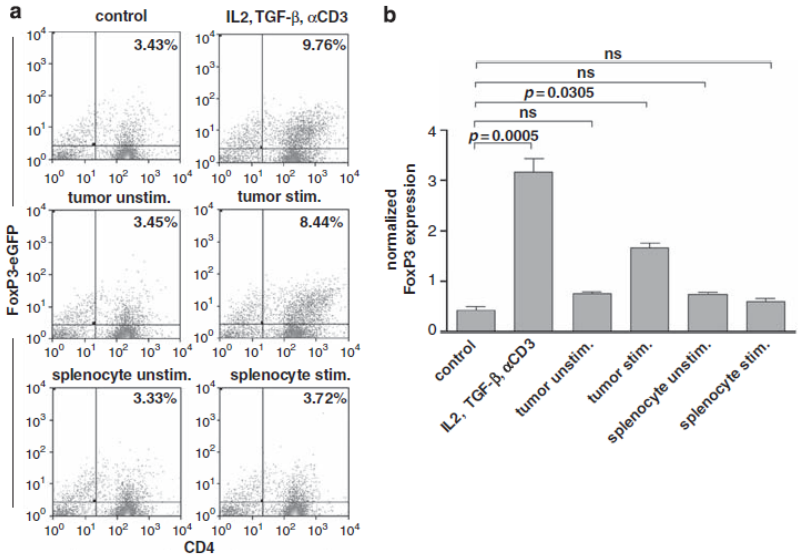

Figure 6 Soluble, MALT lymphoma-derived factors induce Treg conversion from conventional $CD4^+CD25^-$ T cells. (**a**, **b**) $CD4^+CD25^-$ T cells isolated from a mouse expressing enhanced green fluorescent protein (eGFP) under the control of the *foxp3* promoter were cultured for 5 days in medium (control), or with interleukin-2 (IL-2), TGF-β and αCD3, or with supernatants collected from unstimulated or stimulated (*H. felis* extract added) tumor cell suspension cultures. Single-cell suspension cultures of splenocytes isolated from the donor mouse were used as controls. FoxP3 induction was assessed by fluorescence-activated cell sorting (FACS) analysis of eGFP expression of $CD4^+$ cells (**a**) and by FoxP3 real-time reverse transcriptase-PCR (RT-PCR) analysis (**b**). The data shown are representative of two (**a**) and three (**b**) independent experiments.

cases (4.8%), and higher in the gastritis cases (35%, Figure 8b). Interestingly, the Treg population was substantial in many of the low- and high-grade lymphomas, making up on average 33% of the whole $CD4^+$ population in both groups (Figure 8b). Tregs were less abundant in the gastritis cases (9.2% of $CD4^+$); their proportion as a fraction of all $CD4^+$ cells was therefore much closer to the 'normal' ratio of 10–15% of $CD4^+$ cells in the periphery as reported for humans. The accumulation of $FoxP3^+$ cells was further confirmed by quantitative real-time reverse transcriptase-PCR, which we conducted on matched pairs of low-grade MALT lymphoma and corresponding gastritis tissues from seven patients who had been diagnosed with *H. pylori*-positive, translocation-negative gastric MALT lymphoma (Figure 8c). The over-representation of FoxP3 transcript in the tumor biopsy material in comparison to normal inflamed surrounding tissue confirmed the accumulation of Tregs, specifically in the tumors. The RNA isolated from the human lymphoma/gastritis pairs further allowed us to quantify the expression of the Treg-recruiting chemokines CCL17 and CCL22 (Figure 8d). Interestingly, both were expressed significantly higher in tumor tissue compared with corresponding gastritis (Figure 8d). No correlation between the two chemokines was observed, suggesting that they are regulated independently of each other. In conclusion, human low-grade MALT lymphomas are infiltrated by large numbers of $FoxP3^+$ Tregs, which may be recruited to the tumor mass through the same mechanism used by their murine counterparts.

Discussion

We have shown previously that *Helicobacter*-dependent murine MALT lymphomas are infiltrated by large numbers of $CD4^+$ T cells, and that the tumor B cells express functional, rearranged, polyreactive immunoglobulins on their surface.[14] In this study we have assessed the relative contribution of proliferative signals originating from the BCR and from tumor-infiltrating T-helper cells to tumor cell growth *in vitro* and *in vivo*. Several hypotheses addressing the pathogenesis of MALT lymphoma, which had been raised previously based on histopathological observations and, to some extent, experimental manipulation of human biopsy material, were confirmed and extended by our results, and underscore the unique status that MALT lymphomas have among B-cell malignancies with respect to their dependence on external signals.

The availability of definitive clinical evidence proving that low-grade MALT lymphomas regress upon eradication of the causative 'antigen', *H. pylori*,[3,4] led to the postulation of a role for BCR signaling in MALT lymphomagenesis,[11,22–26] and sparked a search for potential tumor antibody targets. Most studies have implicated autoantigens, ranging in diversity from (unspecified) antigens expressed on follicular dendritic cells and post-capillary venules[24] to antigens of plasma cells[26] and auto-IgG.[11] However, the functional significance of antigen binding has not been shown for these targets because of the difficulties in obtaining sufficient amounts of viable tumor cell material for *ex vivo* culturing (surgical biopsies have not been available since *Helicobacter* eradication therapy replaced surgery as the first-line treatment of gastric low-grade MALT lymphoma) and the lack of MALT lymphoma cell lines. We show here that purified tumor B cells isolated from whole tumor cell suspensions proliferate upon exposure to a variety of target antigens, which were shown previously to be recognized by tumor-derived immunoglobulins.[14] Whereas it can be argued that some antibody targets (*H. felis* sonicate and single-stranded DNA) contain Toll-like receptor ligands and therefore could in

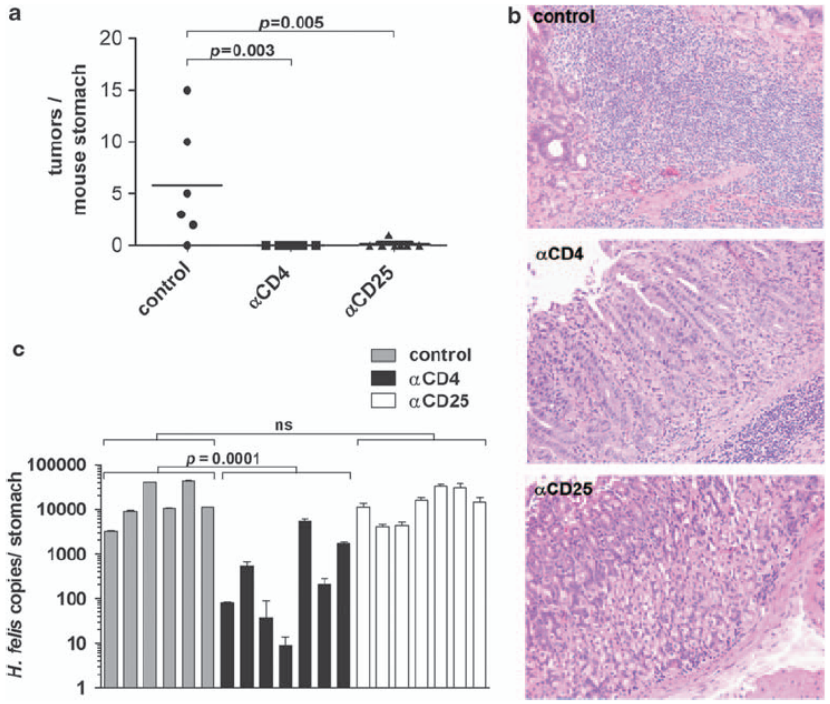

Figure 7 The systemic depletion of $CD4^+$ and $CD25^+$ T cells leads to tumor regression in *H. felis*-infected BALB/c mice. A total of 20 female BALB/c mice were orally infected with *H. felis* at 6 weeks of age and maintained for 18 months in individually ventilated cages. During the last 3 months of the 18-month experiment, two groups received intraperitoneal biweekly injections of antibodies directed against CD4 ($n=7$) or CD25 ($n=7$; 300 μg per dose). Another group ($n=6$) remained untreated. Upon killing, the stomachs were opened and the number of tumors per stomach were recorded (**a**). Horizontal lines represent the means. (**b**) Representative hematoxylin and eosin (H&E)-stained sections of one mouse per group. (**c**) *Helicobacter* colonization was determined for every mouse included in the study using a quantitative PCR protocol amplifying the *flaB* gene of *Helicobacter felis* from whole stomach genomic DNA.

principle trigger proliferation of B cells through crosslinking of Toll-like receptors, this is not the case for other targets tested (gastric mucosal extract and IgG). We deduce from this result that tumor B cells can be stimulated through their BCR. This conclusion is further supported by the intriguing finding that MALT lymphoma immunoglobulins are subject to ongoing somatic hypermutation,[11,13,15] yet avoid class switch recombination,[27] indicating that surface IgM-positive tumor cells are favored by positive or negative selective forces. At the molecular level, the resistance of MALT lymphoma B cells to isotype switching has been explained by their propensity to undergo aberrant class switch recombination,[27] a process by which the IgH locus recombines in a way so that switch regions are deleted and the IgM^+ status is conserved.

Although isolated tumor B cells replicate upon addition of their cognate antigen, their proliferation can be enhanced approximately tenfold by the presence of tumor-infiltrating T cells. This result suggests that MALT lymphoma B cells resemble normal B cells with respect to their reliance on T-cell help. Tumor-infiltrating $CD4^+$ T cells coexpress CD40L and the activation marker CD69 and produce large quantities of T-helper cell type 2 cytokines such as interleukin-4.[7,28] By depleting $CD40L^+$ cells from the cultures, we found that this population was essential for providing B-cell stimulatory signals. However, blocking the direct interaction between CD40L and its receptor did not abrogate tumor cell proliferation. This suggests that soluble activated T cell-derived signals are more important than a direct interaction between both cell types for inducing tumor B-cell proliferation. Our data confirm previous results by Greiner *et al.*[22], who reported CD40L expression on human MALT lymphoma-infiltrating T cells. Greiner *et al.*[28] were able to culture human explanted MALT lymphoma cells in the 'CD40 system', that is, under constant stimulation with agonistic CD40 antibody in conjunction with T-helper cell type 2 cytokines, which led them to conclude that CD40 signaling was essential for tumor B-cell proliferation. In light of our findings it seems that although the tumor B cells can in principle respond to agonistic CD40 stimulation because of high CD40 expression,[7] the direct interaction between both cell types through CD40/CD40L is not required for tumor cell proliferation, at least *in vitro*.

Several groups have reported that tumor-infiltrating T cells are specific for *H. pylori*,[29,30] and have postulated that T cells rather than tumor B cells constitute the *Helicobacter*-specific component of the tumor mass. This assumption fits very well with our model; however, we found that purified T cells from other compartments such as the spleen, which should be only modestly enriched for *H. pylori* reactivity,[16] are also able to significantly enhance tumor cell proliferation.

An unexpected finding of our study was the striking overrepresentation of $FoxP3^+$ Tregs in the tumor-infiltrating T-cell compartment, both in the murine tumors and in human patient

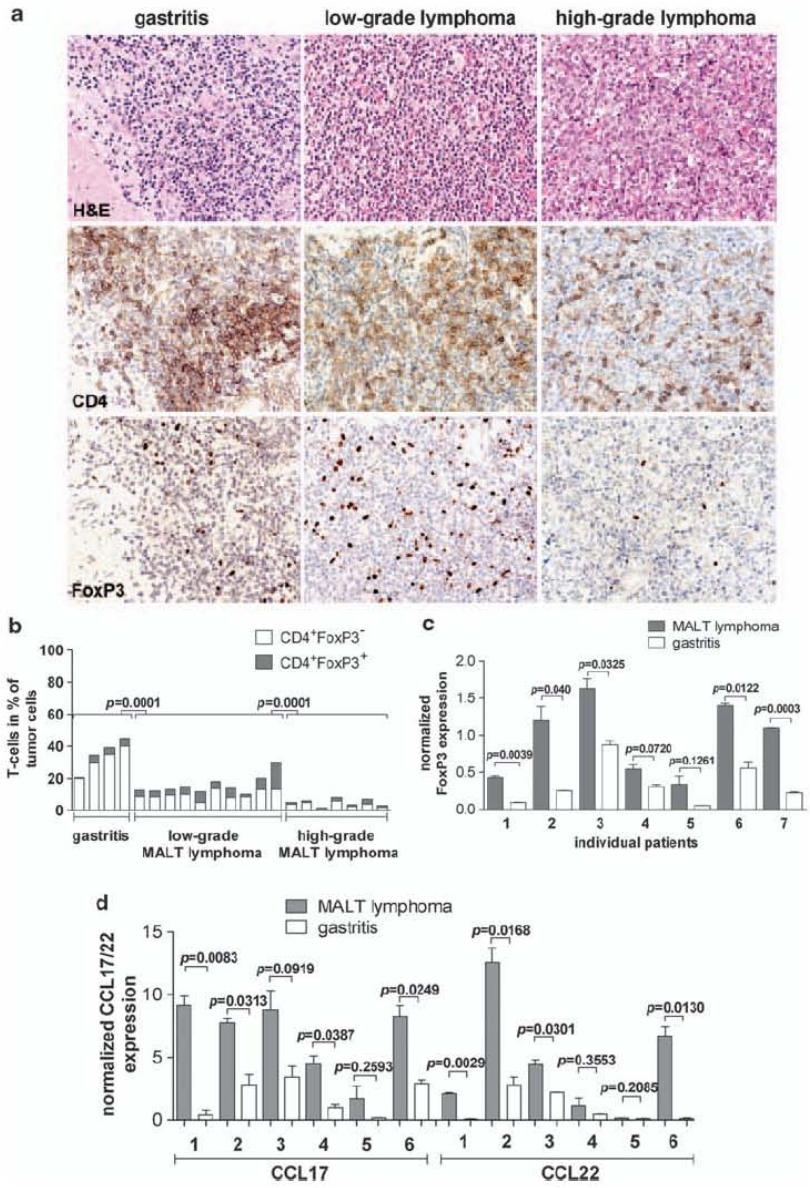

Figure 8 Human low-grade MALT lymphomas are enriched for CD4$^+$ T cells and FoxP3$^+$ Tregs. (**a**, **b**) Consecutive sections of 10 low-grade and 7 high-grade cases of gastric lymphoma were stained with hematoxylin and eosin (H&E) and immunostained for CD4 and FoxP3. Four gastritis cases were also included. Representative stained sections are shown for one case per disease entity in **a**; a quantification of positive cells is shown in **b** for all cases. FoxP3$^+$ cells are shown as a fraction of all CD4$^+$ T cells. At least 500 cells in two and three fields were counted. The *P*-values indicate the significance of differences in CD4$^+$ infiltration. (**c**) Tumor biopsies from an independent set of seven low-grade lymphoma patients were compared with matched histologically confirmed gastritis from the same patients with respect to FoxP3 expression. FoxP3 levels as determined by real-time reverse transcriptase-PCR (RT-PCR) were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. (**d**) The same matched lymphoma/gastritis pairs (patients 1–6) were subjected to real-time RT-PCR for the chemokines CCL17 and CCL22. Again, transcript levels were normalized to GAPDH. Averages of triplicate readings are shown with s.d. for all real-time PCR results.

biopsy material that was compared with the respective adjacent gastritic tissue. Much work in recent years has focused on intratumoral Tregs and their putative role in fostering an immune-privileged niche and blocking antitumor immunity.[18–20]

In our model of *Helicobacter* infection-induced low-grade MALT lymphoma, the depletion of Tregs in the final 3 months of lymphomagenesis reduced the tumor burden as efficiently as the depletion of all CD4$^+$ T cells, presumably by inducing the

regression of pre-existing tumors. This finding was supported by an equally robust effect of Treg depletion on tumor cell proliferation *in vitro*.

Tregs can be recruited, expanded or converted in the tumor microenvironment.[18,19] We found evidence for two distinct mechanisms that MALT lymphoma cells use to enhance Treg infiltration. On one hand, supernatants from actively proliferating (but not resting) tumor cells were capable of inducing Tregs *de novo* from conventional T cells in the absence of a TCR-mediated stimulus. Treg conversion was thus mediated by a soluble factor, but was independent of TGF-β, as addition of a TGF-β-neutralizing antibody did not block conversion (data not shown). On the other hand, tumor cell supernatants efficiently recruited Tregs across transwell filters; again, only the supernatants of proliferating tumor cells had Treg-recruiting activity. Pure populations of malignant tumor B cells, as well as T-cell-depleted tumor cell suspensions, retained much of their ability to recruit Tregs, suggesting that the malignant B cells themselves rather than tumor-infiltrating T cells are the source of Treg chemoattractants in the culture supernatants.

The recruitment of Tregs depended on the two chemokines, CCL17 (also known as thymus and activation-related chemokine, TARC) and CCL22 that, either alone or in combination, had previously been shown to recruit Tregs into epithelial cancers of the stomach[21] and ovaries.[18] Production of these chemokines by malignant B cells has also been shown in follicular lymphoma[31] and in Epstein–Barr virus-associated B-cell lymphoproliferative disorders,[32] although the significance of CCL17 and CCL22 production is less clear in these scenarios. In follicular lymphoma, Treg accumulation in the tumor mass has been attributed mostly to the conversion of Tregs from conventional T cells, which, as in our case, is independent of TCR stimulation and also independent of TGF-β.[31]

Interestingly, we found that intratumoral Tregs were highly suppressive against autologous T cells, more so than natural $CD25^+$ Tregs isolated from the corresponding spleens and mesenteric lymph nodes of the tumor Treg donors. Whether the suppressive activity of intratumoral Tregs is required for their tumor-promoting function cannot be clarified definitively in our experimental setting. However, two observations argue against the notion that low-grade MALT lymphomas benefit from intratumoral Tregs through mechanisms of immune privilege, and instead favor the concept that malignant B cells are directly stimulated by Treg-derived contact-dependent or soluble signals. First, Treg depletion prevents tumor B-cell proliferation *in vitro*, an effect that can hardly be attributed to enhanced antitumor immune responses. Secondly, high-grade transformed gastric lymphomas harbor less, not more, Tregs and overall $CD4^+$ T cells compared with their low-grade counterparts. Therefore, Treg numbers are not a suitable prognostic marker for gastric lymphoma, which has a good prognosis at early (low grade) stages and a poor prognosis upon high-grade transformation. Rather, low numbers of Treg and overall infiltrating T cells are in line with the clinical finding that high-grade lymphomas are antigen independent and cannot be treated by *Helicobacter* eradication therapy because of their acquisition of chromosomal translocations and mutations that affect, among others, the *BCL6* gene.[33,34] Future research will delineate the mechanisms through which Tregs promote tumor B-cell proliferation in the *ex vivo* culture model introduced here; for example, the neutralization of soluble or contact-dependent Treg-derived signals such as interleukin–10, TGF-β or CTLA-4 should clarify whether these are crucial for promoting tumor cell proliferation *ex vivo*.

In summary, our results provide evidence that low-grade MALT lymphoma B cells require at least two independent signals for proliferation *in vitro* and *in vivo*. One signal is received through surface-exposed tumor immunoglobulin, which is polyreactive and binds multiple unrelated self and foreign antigens with similar affinity. The other signal is provided by tumor-infiltrating T cells, which can enhance B-cell growth through contact-independent mechanisms. The overrepresentation of Tregs compared with conventional $CD4^+$ T cells in both the human and murine tumor microenvironment and their active recruitment through tumor B cell-derived chemokines argue in favor of a direct role for this population in stimulating tumor growth. Identification of the microenvironmental molecules critical for tumor growth may lead to specific new intervention strategies for the treatment of MALT lymphoma and other antigen-dependent malignancies.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

3.3 Epigenetic silencing of miRNA-203 dysregulates ABL1 expression and drives *Helicobacter*-associated gastric MALT lymphomagenesis

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Summary: Herein, we endeavored to define a novel molecular pathway involved in the pathogenesis of MALT lymphoma. The miRNA transcriptomes of MALT lymphomas and normal tonsil tissues were first compared at a genome-wide level. One miRNA, *miR-203*, was 11-fold downregulated in the lymphomas and was shown to be silenced due to CpG island promoter hypermethylation. Ectopic re-expression of *miR-203* by transfection of Burkitt's lymphoma cells or lentiviral transduction of primary murine MALT lymphoma cells was sufficient to prevent tumour cell proliferation *in vitro*. We further show here that the miR-203 target, ABL1, is over-expressed in MALT lymphoma compared to matched gastritis material from the same patients. In addition, proliferation of primary MALT lymphoma cells explanted from *Helicobacter*-infected mice could be blocked by the tyrosine kinase inhibitors, imatinib and dasatinib. Finally, the treatment of tumour-bearing mice with imatinib induced MALT lymphoma regression *in vivo*. In summary, our results show that the progression from reactive gastritis to MALT lymphoma may be epigenetically regulated by *miR-203* promoter methylation and ABL1 represents a novel target for the treatment of this malignancy.

Epigenetic silencing of miRNA-203 dysregulates ABL1 expression and drives *Helicobacter*-associated gastric lymphomagenesis

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Running title: Silencing of miR-203 drives MALT lymphomagenesis

Abbreviations: MALT, mucosa-associated lymphoid tissue; miRNA, micro-RNA; LNA, locked nucleic acid.

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Summary

Gastric mucosa-associated lymphoid tissue lymphoma develops in the chronically inflamed mucosa of *Helicobacter pylori*-infected patients. MicroRNA expression profiling of human MALT lymphoma revealed an 11-fold down-regulation of miR-203, which resulted from promoter hypermethylation and coincided with the dysregulation of the miR-203 target ABL1. Demethylating treatment of lymphoma B-cells led to an increase in miR-203 expression and concomitant ABL1 down-regulation. The lentiviral delivery of miR-203, as well as treatment with ABL inhibitors, prevented primary MALT lymphoma cell proliferation *in vitro*. Finally, the treatment of tumor-bearing mice with imatinib induced MALT lymphoma regression *in vivo*. Our results show that MALT lymphomagenesis is epigenetically induced by miR-203 promoter methylation and identify ABL1 as a novel target for the treatment of this malignancy.

Significance

Gastric MALT lymphoma represents an interesting paradigm of bacterially induced tumors. The molecular pathways involved in the clonal outgrowth of malignant B-cells from a background of chronic active *Helicobacter*-associated gastritis remain ill-defined. We show here that this process is epigenetically regulated, and involves the regulatory non-coding RNA miR-203 and its target ABL1. Using human material, various cell culture models and a mouse model of *Helicobacter*-induced lymphoma, we provide evidence that miR-203 is a bona fide tumor suppressor microRNA in the context of MALT lymphomagenesis and that its lentiviral reintroduction suppresses tumor cell proliferation *in vitro*. We further introduce ABL1 as a promising new target for MALT lymphoma treatment based on our finding that ABL inhibitors induce tumor regression in experimentally infected mice.

Highlights

-miR-203 is epigenetically regulated in MALT lymphoma by promoter hyper-methylation
-lentiviral re-expression of miR-203 blocks primary MALT lymphoma cell proliferation
-miR-203 exerts its tumor suppressive activity by repressing its target ABL1
-inhibitors of ABL enzymatic activity prevent MALT lymphoma growth *in vitro* and *in vivo*

Introduction

Mucosa-associated lymphoid tissue (MALT) lymphomas account for 8% of all non-Hodgkin's lymphomas (Thieblemont et al., 2005). The development of gastric MALT lymphoma is tightly linked to chronic infection with the human bacterial pathogen *Helicobacter pylori* (Isaacson and Du 2004); consequently, antibiotic eradication therapy is now the first line treatment for this malignancy (Bertoni et al., 2002, Bertoni and Zucca 2002, Wundisch et al., 2005). MALT lymphomagenesis is initiated by *H. pylori*-associated chronic inflammation and the subsequent accumulation of gastric organized lymphoid tissue, from which individual neoplastic clones may grow out and invade the adjacent epithelium (Isaacson and Du 2004).

We have shown recently that MALT lymphoma tumor immunoglobulins (Igs) are clonal, somatically hypermutated and polyreactive, i.e. they bind to a variety of unrelated self- and foreign antigens (Craig et al., 2010a). The same panel of antigens that are recognized by MALT lymphoma surface Igs induce proliferation of explanted tumor cells, supporting the notion that early low grade gastric MALT lymphoma is an antigen-dependent malignancy (Craig et al., 2010b). Low grade gastric MALT lymphomas are infiltrated by large numbers of T-cells, which are polarized to produce Th2 cytokines such as interleukin-4 (Knorr et al., 1999; Mueller et al., 2005). The depletion of T-cells prevents the proliferation of tumor cells *ex vivo* and induces tumor regression in a mouse model of gastric MALT lymphoma (Craig et al., 2010b), implying a synergistic role for T-cell-derived signals and B-cell receptor-mediated antigen recognition during early MALT lymphoma pathogenesis.

MicroRNAs (miRNAs) are well-conserved, 18-25 nucleotide long non-coding RNAs with pivotal roles in post-transcriptional gene regulation (Ambros et al., 2004). miRNA expression patterns correlate with particular cancer types (Lu et al., 2005) and are predictive of clinical outcome (Calin et al., 2005; Yanaihara et al., 2006). Over 50% of miRNA genes are located in cancer-associated genomic regions (Calin et al., 2004). Many miRNAs are known to function as tumor suppressors, regulating the expression of oncoproteins such as RAS (Johnson et al., 2005) and c-MYC (O'Donnell et al., 2005). Here, we demonstrate that miR-203 is down-regulated in gastric MALT lymphoma due to promoter hypermethylation.

The ectopic re-expression of miR-203 in primary MALT lymphoma cells down-regulates the expression of the miR-203 target ABL1 and blocks tumor cell proliferation *in vitro*. Finally, inhibition of ABL1's tyrosine kinase activity by imatinib blocks MALT lymphoma cell proliferation *ex vivo* and prevents tumor formation in mice, suggesting for the first time an important oncogenic role for ABL1 in the pathogenesis of B-cell tumors not harboring the t(9;22) chromosomal translocation fusing the *bcr* and *abl1* genes.

Results

The expression of miR-203 and of its target ABL1 is dysregulated in MALT lymphoma

In order to identify miRNAs that are differentially expressed in human *Helicobacter*-associated MALT lymphoma compared to normal lymphoid tissue, we generated array-based expression profiles for five low-grade MALT lymphoma and four tonsil samples that included all 795 currently annotated human mature miRNAs. Of the 157 miRNAs exhibiting high variation across the nine samples, six were strongly down-regulated in all cases of lymphoma, but in none of the tonsil samples (Figure 1A, Supplemental Table 1). We focused on miR-203, which was downregulated by ~11 fold in the lymphomas and has previously been implicated in tumor suppression (Bueno et al., 2008). Its dysregulation could be confirmed by quantitative (q)PCR in the samples used for expression profiling as well as in an independent set of archived cases of *Helicobacter*-associated gastritis and gastric MALT lymphoma (Figure 1B,C). As miR-203 is known to regulate ABL1 (Bueno 2008), we assessed ABL1 expression in eight cases of gastric MALT lymphoma for which matched gastritis material was available. qPCR analysis revealed that seven of the eight lymphoma samples exhibited higher ABL1 expression than the corresponding gastritis (Figure 1D). Similar patterns of inverse miR-203 and ABL1 expression were detected in four matching pairs of gastric lymphoma and corresponding gastritis harvested from *Helicobacter*-infected BALB/c mice (Figure 1E,F). In summary, miR-203 and its target ABL1 are differentially regulated in human gastric lymphoma as well as in a mouse model of the disease, implying ABL1 as a possible target of miR-203 in MALT lymphomagenesis.

The miR-203 promoter is specifically hypermethylated in MALT lymphoma

The promoter region of the miR-203 genomic locus contains a CpG island flanking the transcriptional start site (Figure 2A), which has been shown to be hypermethylated in certain hematological malignancies and hepatocellular carcinoma (Bueno et al., 2008; Furuta et al., 2009). We determined the methylation status of the miR-203 promoter in several cases each of normal human tonsil, gastritis and low as well as high grade gastric MALT lymphoma by sodium bisulfite genomic sequencing. The miR-203 promoter region was heavily methylated in the majority of independent clones analyzed of all low and high-grade MALT lymphoma samples, but was largely unmethylated in the tonsil and gastritis tissues

(Figure 2B). In conclusion, the miR-203 promoter appears to be specifically hypermethylated in MALT lymphoma, which may lead to silencing of the genomic locus and may contribute to MALT lymphomagenesis.

The expression of miR-203 is epigenetically regulated and controls ABL1 levels and lymphoma cell proliferation *in vitro*

To determine whether promoter methylation indeed affects miR-203 transcription, and as a consequence, ABL1 expression, we took advantage of a non-Hodgkin's lymphoma cell line exhibiting a degree of miR-203 promoter methylation that is comparable to that found in human MALT lymphoma (BL2; Figure 3A). The treatment of BL2 cells with the DNA-demethylating agent 5'-azacytidine (Aza), either alone or in combination with the histone deacetylase inhibitor 4-phenylbutyric acid (PBA), resulted in decreased miR-203 promoter methylation and a concomitant rise in miR-203 expression (Figure 3A,B); the expression of ABL1 was inversely correlated with miR-203 at the mRNA and protein levels (Figure 3C,D). The combined treatment with both compounds resulted in a more efficient drop in promoter methylation and a greater increase in miR-203 expression, but did not further reduce ABL1 expression (Figure 3A-D). To confirm directly that miR-203 targets ABL1, pre-miR-203 precursor molecules were introduced into BL2 cells by electroporation. The ectopic expression of pre-miR-203 led to a significant down-regulation of ABL1 expression at both the mRNA and the protein levels as compared to a scrambled negative control pre-miR (Figure 3E,F) and blocked the proliferation of BL2 cells as determined by [^{3}H] thymidine incorporation (Figure 3G). The combined results suggest that miR-203 is epigenetically regulated and acts as a tumor suppressor miRNA through its effects on ABL1 expression.

The proliferation of MALT lymphoma cells is blocked by imatinib *in vitro* and *in vivo*

To assess the effects of miR-203 delivery on primary murine MALT lymphoma cells, we generated lentiviral particles carrying a miR-203-encoding expression vector and transduced primary murine gastric MALT lymphoma cells isolated from BALB/c mice that had been infected with *H. felis* for 18 months. The lentiviral delivery of miR-203 to explanted murine MALT lymphoma cells resulted in increased miR-203 levels, decreased

ABL1 expression and a concomitant block in the *Helicobacter* antigen-dependent proliferation of the cells (Figure 4A-C). These observations raised the possibility that ABL inhibitors such as imatinib (Gleevec) might prevent the proliferation of primary MALT lymphoma cells *in vitro* and *in vivo*. Indeed, addition of imatinib to the primary cell cultures efficiently blocked their proliferation in a dose-dependent manner (Figure 4D); similar results were obtained with the ABL inhibitor dasatinib (Figure 4D). Tumor cells that had spread to the spleen were equally sensitive to ABL inhibition as cells derived from the primary gastric tumor (Figure 4E). To assess a possible therapeutic effect of imatinib in a preclinical model of *Helicobacter*-induced MALT lymphoma, BALB/c mice were infected for 15 months with *H. felis* to allow for lymphoma development. A group of mice received imatinib via the drinking water for another three months while all other mice remained untreated. Whereas between 2 to 15 tumors had formed in the majority of control mice, all imatinib-treated mice were tumor free as assessed macroscopically and histopathologically at the 18 month post infection endpoint of the study (Figure 4F,G). Imatinib had no effect on *H. felis* colonization densities (Figure 4H), ruling out a direct effect of the treatment on the underlying infection. The combined results suggest that miR-203 re-expression in murine primary MALT lymphoma cells prevents their proliferation and that this effect is mediated by the tyrosine kinase activity of the miR-203 target ABL1 *in vitro* and *in vivo*.

Discussion

Our array-based miRNA transcriptome analysis identified miR-203 as one of the most strongly down-regulated miRNAs in gastric lymphoma compared to normal lymphoid tissue; its dysregulation could be attributed to extensive promoter hypermethylation. Our results further suggest that the loss of miR-203 expression and the resulting dysregulation of its target ABL1 contribute directly to gastric lymphomagenesis and identify ABL1 as a new target in the treatment of this malignancy. Several previous reports have indicated a tumor suppressive role for miR-203. It is encoded on a fragile 7Mb region of murine chromosome 12 that encodes ~12% of all genomic miRNAs and that is frequently deleted in hematopoietic malignancies (Bueno et al., 2008). Its expression is downregulated in cancers of the liver (Ladeiro et al., 2008), central nervous system (Gaur et al., 2007) and in some types of leukemia (Bueno et al., 2008). Experimental inhibition of miR-203 enhances the growth of lung carcinoma cells (Cheng et al., 2005) and the restoration of miR-203 levels significantly reduces the proliferation of hepatocellular carcinoma (Furuta et al., 2009), certain leukemias such as chronic myelogenous leukemia (CML) (Bueno et al., 2008) and head and neck squamous cell carcinoma (Lena et al., 2008).

MALT lymphomas are negative for the Philadelphia chromosome (Ph^-, data not shown), i.e. they do not harbor the t(9;22) reciprocal translocation fusing the BCR and ABL1 gene loci that is a hallmark of CML and of a subset of acute lymphoblastic leukemias (ALL) (Ottmann and Wassmann 2005). However, we found that ABL1 is significantly more strongly expressed in human MALT lymphoma biopsies than in corresponding gastritis material from the same patient. The treatment of BL2 cells (which are also Ph^- and harbor a methylated miR-203 promoter region) with demethylating agents and consequent re-expression of miR-203 repressed cellular ABL1 levels and reduced BL2 proliferation, showing that miR-203 can function as a tumor suppressor independently of the t(9;22) translocation.

ABL1 is predominantly expressed in the hematopoietic system, in particular in lymphocytes; targeted deletion or mutation of ABL1 in mice results in splenic and thymic atrophy and lymphopenia as well as an increased susceptibility to infections (Schwartzberg et

al., 1991). In B-cells, ABL1 functions in B-cell receptor signaling, probably by directly interacting with, and phosphorylating, the BCR co-receptor CD19 (Zipfel et al., 2000). ABL1 overexpression has been associated with Ph^- hematopoietic malignancies such as chronic lymphocytic leukemia (CLL) (Lin et al., 2006). In CLL, ABL1 overexpression has been linked to constitutively active BCR signaling and NF-κB activation (Lin et al., 2006), i.e. to a signaling pathway that is known to contribute to MALT lymphomagenesis (Isaacson and Du 2004).

We have shown recently that BCR signaling synergizes with T-cell-derived signals to drive MALT lymphoma cell proliferation (Craig et al., 2010b). Inhibition of ABL1 expression by miR-203 replacement or inhibition of its tyrosine kinase activity would therefore be predicted to block the *Helicobacter*-induced proliferation of primary MALT lymphoma cells. Indeed, the lentiviral delivery of miR-203 or treatment of explanted murine MALT lymphoma cells with the tyrosine kinase inhibitor imatinib efficiently prevented their proliferation. The treatment of *Helicobacter*-infected mice prevented MALT lymphomagenesis *in vivo*, suggesting that ABL inhibition might be a valid strategy for the treatment of patients that are refractory to eradication therapy. In addition to its beneficial effects in patients with CML (Baselga et al., 2006), imatinib has recently shown promise in adult patients with Ph^+ B-cell ALL (Ottmann and Wassmann 2005) and has been proposed for the treatment of Ph^- CLL patients overexpressing ABL1 (Lin et al., 2006).

In summary, we show here that the progression from reactive, *Helicobacter*-associated gastritis to low grade MALT lymphoma is likely to be epigenetically regulated through methylation of the miR-203 promoter region. Transcriptional repression of miR-203 results in dysregulation of ABL1, which in turn drives MALT lymphoma cell proliferation. Our results identify ABL1 as a promising new target for the treatment of low grade MALT lymphoma, in particular of the ~20% of patients who are refractory to eradication therapy as a first line treatment of the disease.

Experimental procedures

Patient material

Human patient material was obtained from eight patients with *H. pylori*-positive, t(*11*;*18*)(q21:q21)-negative gastric low grade MALT lymphoma that were part of a previously published study conducted at Philipps-University Hospital Marburg, Germany (Huynh et al., 2008). miRNA expression analysis was performed on fresh and archived cases of *H. pylori*-positive gastritis, *H. pylori*-positive gastric low or high grade MALT lymphoma and tonsil material drawn from the surgical pathology files of the Institute of Pathology at the Cantonal Hospital St. Gallen, Switzerland. All data were blinded to guarantee patients' protection and were generated in agreement with the guidelines for use of human material in research issued by the participating Institutions' Ethics Committees.

Animal experimentation, cell culture, nucleoporation and lentivirus infection

Female BALB/c mice were infected intragastrically at six weeks of age with $5x10^7$ *H. felis* (CS1, ATCC 49179). All procedures were approved by the Zurich cantonal veterinary office. Mice received 75mg/kg per day imatinib in their drinking water for the final 3 months of an 18 month infection experiment. After 18 months, macroscopically visible gastric tumors were collected, single cell suspensions were generated and cultured for 3 days in RPMI/10 % FCS with 10 μg/ml *Helicobacter* lysate and 0.01-30 μM imatinib or dasatinib (generously provided by Novartis, Basel, Switzerland and C. Nevado, Institute of Organic Chemistry, University of Zurich, respectively). Tumor cell proliferation was quantified by [^{3}H] thymidine incorporation. For DNA demethylation, BL2 cells were treated with 5 μM 5'-azacytidine and/or 3 mM 4-phenylbutyric acid (both from Sigma-Aldrich). For the purpose of miR-203 re-expression, $1x10^6$ BL2 cells were nucleoporated using an Amaxa Nucleoporator (Gaithersburg, MD) with miR-203 precursor or negative control oligonucleotides (both from Ambion, Austin, TX) and harvested 48 hours later for ABL1 expression analysis by qRT-PCR or Western Blot as described in the supplemental methods. Primary MALT lymphoma cells were transduced with lentiviral particles harboring a miR-203 expression construct (Systems Biosciences, Mountain View, CA) in the presence of 8

μg/ml polybrene by spinoculation. The production of lentiviruses and the transduction protocol is described in the supplemental methods.

Microarray-based miRNA expression profiling and bisulfite genomic sequencing

Microarray experiments were performed using the Agilent Human miRNA Microarray Kit version 10.0. as described in detail in the supplemental methods. Normalization and statistical analysis was performed using the quantile normalization implemented in the package PreprocessCore and the package genefilter, respectively. All raw data of the microarray experiments are publicly accessible under the accession number GSE23877 (http://www.ncbi.nlm.nih.gov/geo/). The procedures used for determination of the miR-203 promoter methylation status are described in the supplemental methods.

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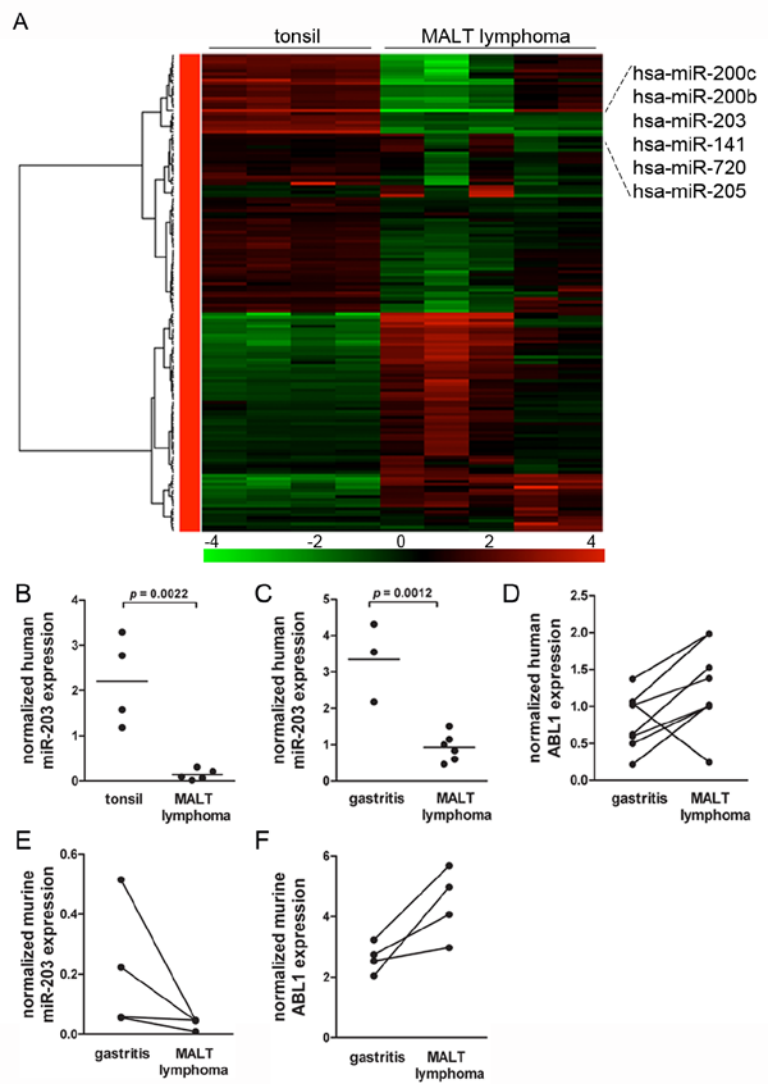

Figure 1. miR-203 and its target ABL1 are dysregulated in MALT lymphoma.

(A) Heatmap representation of 157 miRNAs with a standard deviation of $\log_2$ expression >0.05 across the four tonsil and five MALT lymphoma samples analyzed on Agilent miRNA microarrays; six consistently down-regulated miRNAs are annotated. (B) Validation of miR-203 expression of the samples shown in A as assessed by LNA qRT-PCR. miR-203 levels were normalized to U6 snRNA expression. (C) Expression levels of miR-203 in human gastritis and MALT lymphoma FFPE tissue samples as determined by LNA qRT-PCR. (D) ABL1 expression in MALT lymphoma and corresponding gastritis material derived from eight patients. ABL1 transcript levels were quantified by qRT-PCR and normalized to GAPDH expression. (E,F) miR-203 and ABL1 expression in four pairs of murine MALT lymphomas and corresponding gastritis harvested from 18 month infected BALB/c mice.

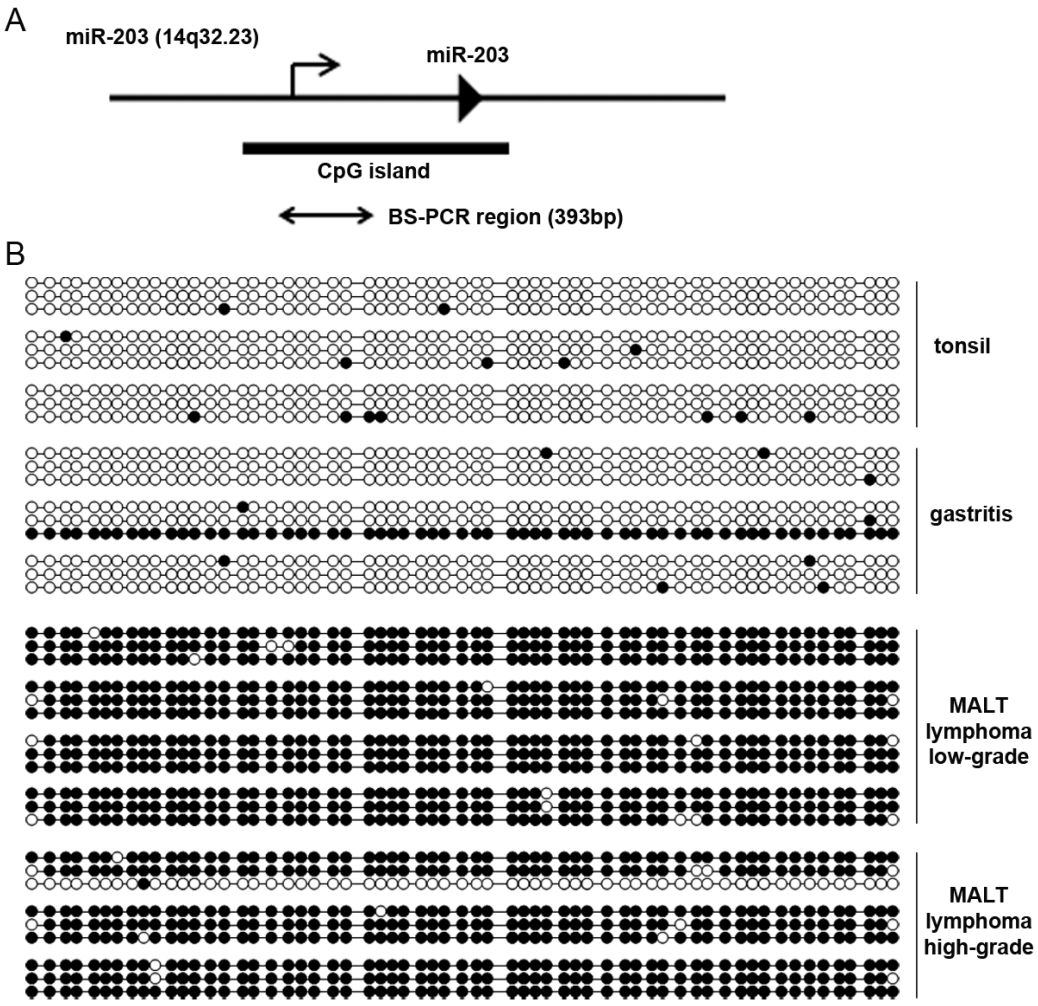

Figure 2. The miR-203 promoter is specifically hypermethylated in MALT lymphoma. (A) Schematic representation of the *miR-203* gene embedded in a CpG island showing the 393 bp region analyzed for methylation status. The position of the *miR-203* gene is indicated by a triangle. The transcription start site is represented by a bent arrow. (B) Bisulfite sequencing of the upstream region of *miR-203* in human tonsil, gastritis and low and high grade MALT lymphoma samples. 3-4 representative samples are shown for each tissue type and 3 single clones are represented for each individual sample. Black and white circles represent methylated and unmethylated CpG, respectively. All 61 sequenced CpGs are indicated.

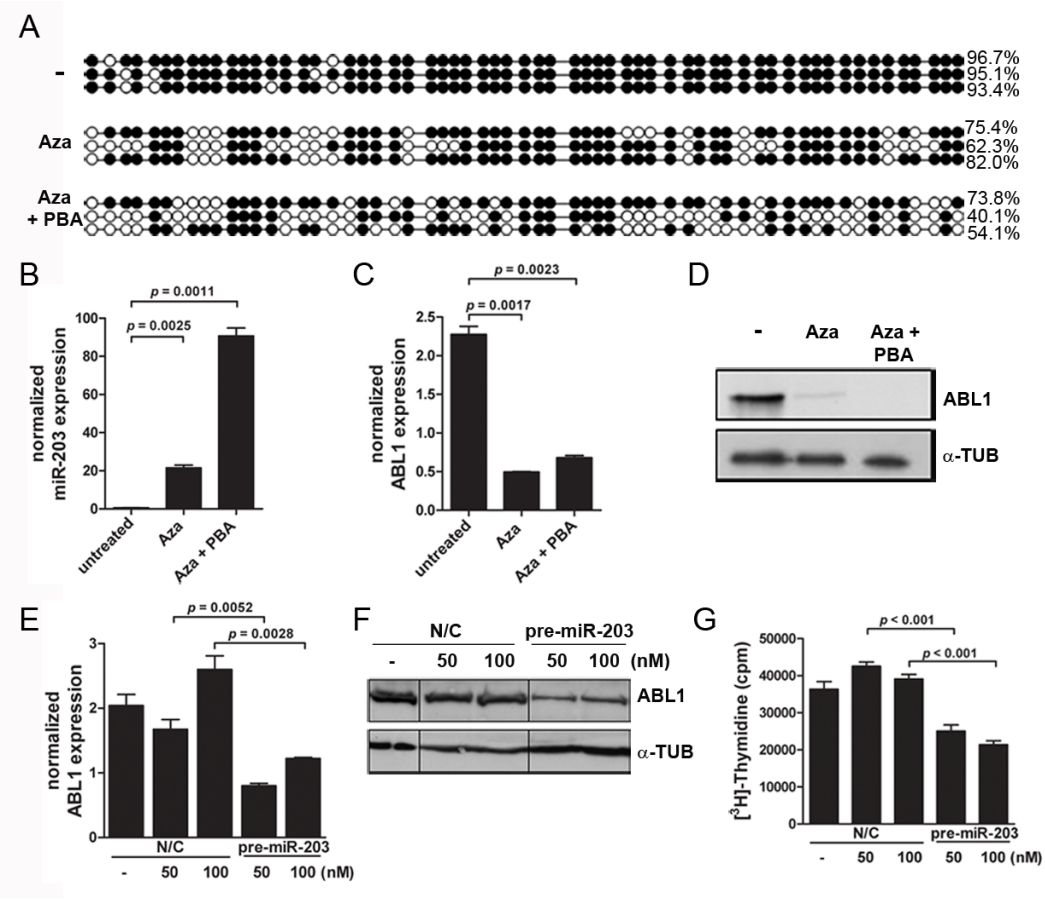

Figure 3. The expression of miR-203 is regulated epigenetically and controls ABL1 expression and lymphoma cell proliferation. (A) miR-203 promoter methylation in BL2 cells as determined by bisulfite sequencing. Cells were either treated with 5 µM 5'-azacytidine, alone or in combination with 3 mM 4-phenylbutyric acid, or left untreated. 3 single clones were sequenced for each treatment and the percentage of methylated CpG dinucleotides is shown. Black and white circles represent methylated and unmethylated CpG. (B,C) qRT-PCR analysis of miR-203 and ABL1 transcript in BL2 cells with and without drug treatment. (D) ABL1 protein levels of the experiment outlined in A-C as assessed by Western blot analysis with α-tubulin serving as a loading control. (E) Normalized ABL1 expression of BL2 cells transfected with either miR-203 precursor molecules (miR-203) or negative control precursor molecules (N/C) at 50 nM or 100 nM final concentration. (F) ABL1 protein levels of the experiment described in E as assessed by Western blot analysis with α-tubulin serving as loading control. (G) The proliferation of cells transfected as described in E as determined by [^{3}H] thymidine incorporation.

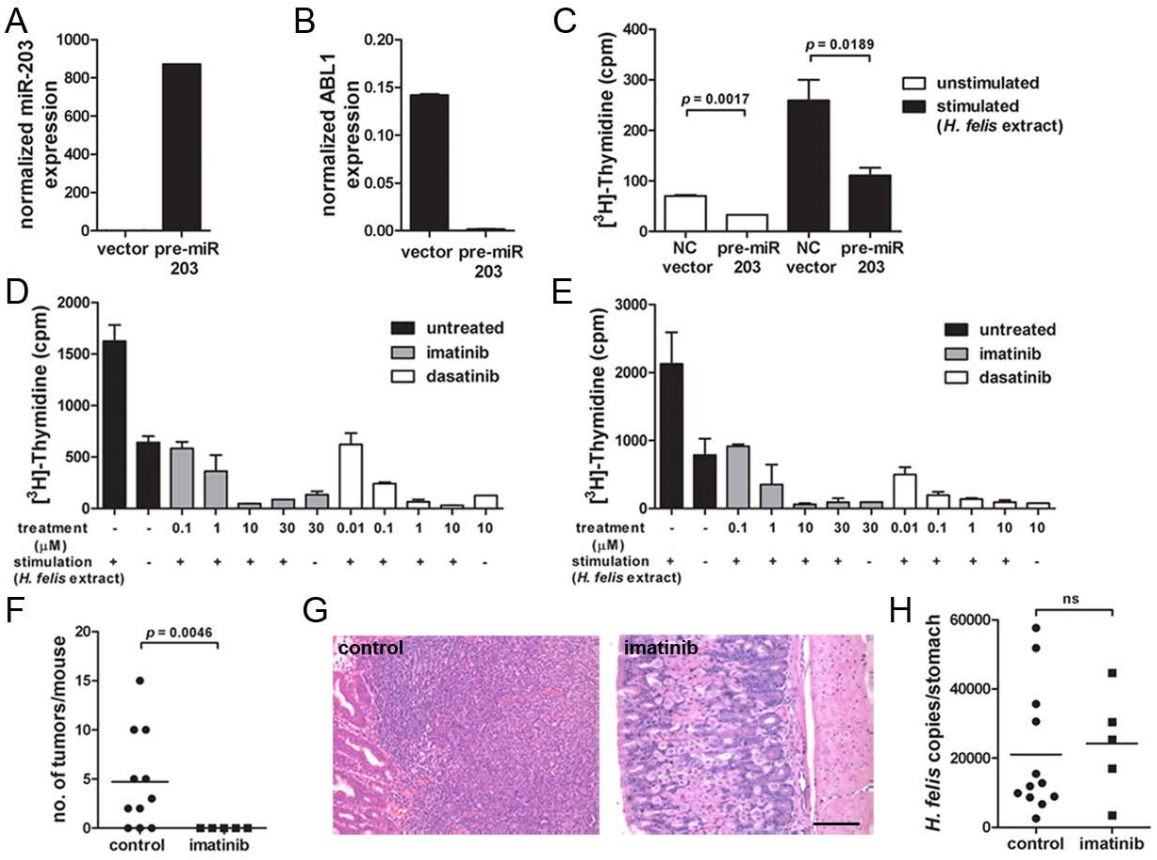

Figure 4. miR-203 replacement and pharmacological ABL inhibition block MALT lymphoma growth *in vitro* and *in vivo*. (A-C) Primary murine MALT lymphoma cells were transduced with lentiviruses carrying a pre-miR-203-expressing or empty vector. miR-203 and ABL1 expression were assessed by qRT-PCR; the proliferation of transduced cells was determined by [^{3}H] thymidine incorporation with or without stimulation with *H. felis* sonicate. (D,E) [^{3}H] thymidine incorporation of gastric (D) and splenic (E) primary murine MALT lymphoma cells stimulated with *H. felis* sonicate and treated with increasing concentrations of imatinib or dasatinib as indicated. Unstimulated cells are included for comparison. Vertical bars indicate standard deviations. (F) Gastric MALT lymphoma formation in female BALB/c mice infected for 18 months with *H. felis*. One group received imatinib through the drinking water for months 16-18 of the experiment. Macroscopically visible tumors >1mm in diameter are plotted. (G) Representative micrographs of H&E-stained sections of the mice shown in F. Scale bar indicates 50 μm. (H) *H. felis* colonization as determined by *flaB*-specific qPCR of the mice shown in F.

Supplemental methods

miR-203 and ABL1 expression analysis

Expression of mature miR-203 was analyzed using the miRCURY locked nucleic acid (LNA) microRNA PCR system following the manufactures' protocol (Exiqon). Briefly, 10 ng of total RNA was subjected to cDNA synthesis using miR-203 or U6 snRNA-specific primers. The cDNA template was diluted 1:10 and real time PCR reactions were performed following the manufactures' recommendations (LightCycler; Roche, Basel, CH). Calculations of miRNA expression levels were performed using the comparative $\Delta\Delta C_t$ method and normalized against U6 snRNA levels. ABL1-specific real time RT-PCR (LightCycler; Roche, Basel, CH) was performed with the LightCycler 480 SYBR Green I master kit (Roche). GAPDH transcript levels were determined for normalization. Human and murine ABL1 primers were published previously (Alvarez et al. 2004; Sun et al., 2001). Protein extracts were made using either RIPA cell lysis buffer (50 mM Tris-HCl [pH7.5], 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1 mM PMSF) or 2x Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris [pH 6.8]). Proteins were separated by SDS/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were probed with antibodies against ABL1 (BD Biosciences, CA, USA) and α-tubulin (Sigma-Aldrich, St. Louis, MO).

DNA methylation analysis of the miR-203 promoter

Specific oligonucleotides for bisulfite sequencing of the miR-203 CpG island were described previously (Bueno et al. 2008). Genomic DNA was isolated from FFPE tissue or fresh material using the RecoverAll total RNA Isolation kit (Ambion, Streetsville, Canada) or the Quiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), respectively. gDNA (2µg) was converted with sodium bisulfite as previously reported (Frommer et al.1992) Following amplification of the bisulfite-converted DNA, the methylation status was assessed by sequencing the miR-203 bisulfite converted promotor region.

Lentivirus production and infection

The pre-mir-203 lentiviral expression construct used to re-express mature mir-203 in murine primary lymphoma cells was purchased from Systems Biosciences (Mountain View, CA). Third-generation lentiviral vector packaging constructs were generously provided by Stefano Ferrari at our Institute and consisted of the three plasmids pMDLg/pRRE, pHCMV-G and pRSVrev. The pre-mir-203 expression vector (2.5 μg) was transfected along with the lentivirus packaging plasmids (2.5 μg of each plasmid) into HEK293T cells and the supernatent was collected 48 and 72 hours after transfection. Primary lymphoma cells were incubated with the viral particles. Empty lentivirus was used as a control for the experiments. *H. felis* stimulated primary MALT lymphoma cells were transduced in the presence of polybrene (8 μg/mL) by spinoculation in a centrifuge at 700 x g for 90 minutes at room temperature in a 96-well plate. 72 hours following infection, tumor cell proliferation was quantified by [^{3}H] thymidine incorporation assay. Expression of mature mir-203 was validated by real-time PCR.

Microarray-based miRNA expression profiling and bisulfite genomic sequencing

Total RNA (including miRNA) was extracted from fresh frozen and FFPE biopsy samples (three 20 μm slices) using the RecoverAll total RNA Isolation kit (Ambion, Streetsville, Canada). RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). miRNA microarray experiments were performed using the Agilent Human miRNA Microarray Kit version 10.0. For each sample, 100 ng total RNA were hybridized with the miRNA array and further processed according to the Agilent's miRNA Microarray System protocol. The arrays were scanned with an Agilent Technology G2565B scanner (Agilent Technologies, Palo Alto, CA). The scanned images were gridded and analysed using Agilent Feature Extraction Software version 9.5. Normalization and statistical analysis was performed with R/Bioconductor. Specifically, we used the quantile normalization implemented in the package PreprocessCore and ran the statistical test using the package genefilter.

3.4 MYC-mediated repression of microRNA-34a promotes high grade transformation of gastric lymphoma by dysregulation of FOXP1

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Summary: This publication represents our effort to reveal aspects of the molecular detail govering the elusive transformation process of MALT lymphoma to gDLBCL. The miRNA expression profiles of these two disease entities were compared and a MYC-induced miRNA profile was found to characterise gDLBCL. Indeed, MYC overexpression was more stongly correlated with gDLBCL than low grade MALT lymphoma cases and the re-expression of certain MYC-associated miRNAs significantly reduced the proliferation of DLBCL cells. In particular, miR-34a showed the strongest anti-proliferative properties. We attribute the tumour suppressive effects of miR-34a to dysregulation of its target FOXP1. Accordingly, FOXP1 overexpression was detected in gDLBCL, but not in low grade lymphoma and transient FOXP1 knock down efficiently blocked DLBCL proliferation. Taken together, our findings elucidate a novel pathway promoting high grade lymphomagensis and suggest miR-34a replacement therapy as a potential therapeutic modality in the treatment of DLBCL.

Myc-mediated repression of microRNA-34a promotes high grade transformation of gastric B-cell lymphoma by dysregulation of FoxP1

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Abstract

Gastric marginal zone B-cell lymphoma of MALT arises in the context of chronic inflammation induced by the bacterial pathogen *Helicobacter pylori*. Such low grade lymphoma may progress by acquiring one of several characteristic chromosomal translocations or by transforming into high grade gastric diffuse large B-cell lymphoma (gDLBCL). The mechanism of malignant transformation remains poorly understood. By comparing microRNA expression profiles of low and high grade gastric lymphoma cases we have identified a signature of ~25 dysregulated microRNAs that share the characteristic of being transcriptionally repressed by Myc. Correspondingly, Myc over-expression was detected in 80% of high grade, but only 20% of low grade lymphomas spotted on a tissue microarray. The siRNA-mediated knock-down of Myc blocked proliferation of DLBCL cell lines. Of the Myc-repressed miRNAs downregulated in malignant lymphoma, miR-34a showed the strongest anti-proliferative properties when overexpressed in DLBCL cell lines. We could further attribute miR-34a's tumor suppressive effects to dysregulation of its target FoxP1. FoxP1 over-expression was detected in gDLBCL, but not in low grade lymphoma; FoxP1 knock-down efficiently blocked DLBCL proliferation. In conclusion, our results elucidate a novel Myc- and FoxP1-dependent pathway of transformation and suggest miR-34a replacement therapy as a promising strategy in lymphoma treatment.

Introduction

Gastric marginal zone B-cell lymphomas of mucosa-associated lymphoid tissue (MALT) lymphomas are low grade extranodal B-cell lymphomas that arise in the context of chronic gastric inflammation induced by persistent *Helicobacter pylori* infection (Parsonnet et al., 1994). In its early stages, MALT lymphoma is an indolent and localized disease that can be treated by antibiotic eradication therapy targeting the underlying infection (Isaacson and Du, 2004). In line with the concept that gastric MALT lymphomas are antigen-driven tumors, the surface immunoglobulins of MALT lymphoma B-cells are clonal, somatically hypermutated, and have undergone positive selection (Qin et al., 1995). We have shown recently that MALT lymphoma tumor immunoglobulins (Igs) are polyreactive, i.e. they bind with similar affinity to various unrelated self and foreign antigens, and show a biased use of Ig V_H gene segments previously linked to poly- and autoreactive antibodies (Craig et al., 2010a). Early MALT lymphomas further require T-cell help in the form of soluble T-helper cell-derived signals, most likely B-cell mitogenic cytokines such as IL4 and IL5 (Craig et al., 2010b).

Low grade MALT lymphomas may progress either through the acquisition of one of three characteristic chromosomal translocations resulting in the constitutive activation of the NF-κB signaling pathway (Isaacson and Du, 2004), or through the histologically evident transformation into high-grade gastric diffuse large B-cell lymphoma (gDLBCL) (Chan et al., 1990; Peng et al., 1997). Such high grade transformation of *Helicobacter*-associated MALT lymphoma accounts for the majority of gDLBCL cases, whereas primary gDLBCL is rare (Chan et al., 1990; Peng et al., 1997). gDLBCL is characterized by antigen-independent growth, resistance to *Helicobacter* eradication therapy and a number of genetic alterations that may contribute to high grade transformation (Starostik et al., 2002). In particular, *TP53* mutations (Du et al., 1995), Bcl6 overexpression (Omonishi et al., 1998) and the aberrant DNA hypermethylation of tumor suppressor genes (Kondo et al., 2009) have been shown to be associated with high grade transformation. However, the precise molecular mechanisms underlying the transition from low-grade MALT lymphoma to gDLBCL remains largely unclear.

MicroRNAs (miRNAs) are an abundant class of small non-coding RNAs, which modulate the expression of their target genes at the post-transcriptional level. Aberrant expression of specific miRNAs has been associated with both solid and hematopoietic malignancies (Lu et al., 2005), including chronic lymphocytic leukemia (Calin et al., 2004a), lung cancer (Calin and Croce, 2006; Yanaihara et al., 2006) and ovarian cancer (Calin et al., 2004b; Zhang et al., 2008). The majority of human miRNAs are located at fragile sites or cancer associated genomic regions (Calin et al., 2004b). For example, the frequent down-regulation of the fragile region encoding mir-15a and mir-16-1 promotes CLL through dysregulation of the Bcl2 oncogene (Calin et al., 2004a). The widespread deregulation of the miRNA transcriptome appears to be a hallmark of cancer and has been attributed to deletions, amplifications or mutations of miRNA loci (Calin et al., 2004b; Zhang et al., 2008), epigenetic silencing (Saito et al., 2006), or the aberrant transcriptional regulation of miRNA genes (Zhang et al., 2008). Several studies have revealed the potential of miRNA expression profiles as diagnostic and prognostic markers of cancers (Yanaihara et al., 2006), which may be more useful than expression analysis of protein-coding genes for the classification and stratification of cancer subtypes (Lu et al., 2005).

Here, we have used a microarray approach to identify miRNAs that are differentially regulated in gastric low grade MALT lymphoma and its transformed high grade disease counterpart. Interestingly, we found that a characteristic set of Myc-repressed miRNAs was down-regulated in the high grade, but not the low grade cases studied. Aberrant Myc expression indeed correlated with high grade transformation as analyzed immunohistochemically using a gastric lymphoma tissue microarray. Bioinformatic target prediction combined with functional analyses revealed that one of the miRNAs found to be down-regulated in high grade gDLBLC, miR-34a, represents a bona fide tumor suppressor miRNA in gastric lymphoma. MiR-34a acts through post-transcriptional control of its direct target FoxP1, a hematopoietic oncoprotein overexpressed in gDLBCL. In conclusion, our findings identify a new mechanism that links the aberrant expression of Myc and the resulting repression of the tumor suppressor miRNA miR-34a to FoxP1 deregulation in high grade transformation of gastric B-cell lymphoma.

Results and Discussion

Myc-repressed miRNAs are specifically down-regulated in high grade-transformed gastric lymphoma

To obtain global miRNA expression signatures of gDLBCL and its precursor lesions, total RNA isolated from 7-8 cases each of *Helicobacter*-associated reactive gastritis, low grade MALT lymphoma and high grade gDLBCL was hybridized to Agilent miRNA microarrays representing 795 human mature miRNAs. Unsupervised hierarchical clustering analysis revealed a clear segregation of the gDLBCL cases from the low grade MALT lymphomas and the gastritis samples (Figure 1A). The segregation of the latter two disease entities was incomplete (Figure 1A), reflecting the relative biological similarity of gastritis and low grade lymphomas. Statistical analysis of the dataset revealed only 25 differentially expressed miRNAs between these two groups ($p<0.05$; suppl. Table 1), whereas 88 miRNAs exhibited significant differences in expression between low and high grade lymphomas (suppl. Table 2).

In order to identify miRNAs with a putative tumor suppressive role in high grade transformation, we focused on the subset of 57 miRNAs that were down-regulated in high grade, but not low grade tumors (Figure 1A). Interestingly, a large fraction of the down-regulated miRNAs had previously been reported to be repressed by the Myc transcription factor (Chang et al., 2008). In fact, we found that of the 24 known human Myc-repressed miRNAs, 21 were down-regulated by at least 1.6- and up to 8.7 fold in high grade vs. low grade gastric lymphomas. The differential expression of two selected miRNAs, let-7 and miR-34a, was verified on the same panel of samples by locked nucleic acid (LNA) real time RT-PCR (Figure 1B,C). In conclusion, we found using whole-genome based miRNA expression profiling that high grade transformation of gastric MALT lymphoma is accompanied by a characteristic signature of repressed miRNAs, a substantial number of which are encoded by known target genes of Myc.

Myc is over-expressed in high grade gastric DLBCL and controls DLBCL proliferation *in vitro*

Having obtained indirect evidence of Myc expression in gDLBCL, but not low grade MALT lymphoma, we next aimed to assess the Myc expression status of a set of 37 gDLBCL and 39 low grade lymphomas spotted onto a gastric lymphoma tissue microarray (Bernasconi et al., 2008). Indeed, 80% of gDLBCL, but only 20% of low grade lymphomas showed high expression of Myc (Figure 2A,B), indicating that Myc expression may be a useful marker for the differential diagnosis of both disease entities. To assess a possible causal link between Myc expression and miRNA down-regulation in DLBCL, we transiently knocked down Myc expression in two DLBCL lines, of which one had the characteristics of the 'activated B-cell' type of DLBCL ('ABC'; U2932) and the other had typical 'germinal center' type features ('GC', SUDHL4). The transient knock-down of Myc indeed increased expression of both let-7 (Figure 2C) and miR-34a (Figure 2D) in both cell lines in relation to a scrambled siRNA. Interestingly, the proliferation of both cell lines as determined by [^{3}H] thymidine incorporation was significantly reduced upon siRNA-mediated Myc knock down (Figure 2E,F), indicating that Myc expression drives lymphoma cell proliferation, possibly via down-regulation of tumor suppressive miRNAs.

To determine which of the Myc-repressed miRNAs have tumor suppressive properties in DLBCL cell lines *in vitro*, we focused on a panel of six miRNAs that were consistently predicted by both the TargetScan and PicTar algorithms to target known or putative hematopoietic oncogenes such as Bcl6, Ezh2, FoxP1 and Pax5. We introduced synthetic, chemically modified double-stranded precursor molecules of the six miRNAs (so-called pre-miRs™) into U2932 and SUDHL4 cells by nucleoporation, either alone or in combination (Figure 2G,H). While all miRNAs on our panel had suppressive effects on tumor cell proliferation in relation to an unspecific negative control miRNA, one candidate, miR-34a, was particularly effective in this respect (Figure 2G,H). miR-34a is a known tumor suppressor miRNA in prostate and lung cancer and is a lead candidate for miRNA replacement therapy for the treatment of these malignancies (Wiggins et al., 2010). In conclusion, our combined bioinformatic and experimental approach identified a miRNA with interesting tumor suppressive characteristics in DLBCL *in vitro*.

miR-34a targets the transcription factor FoxP1 in DLBCL

Bioinformatically predicted targets of miR-34a include the transcription factors Bcl6 and FoxP1, which harbor one and two putative miR-34a seed regions in their 3' untranslated region (UTR), respectively. As both have previously been linked to the pathogenesis of gDLBCL (Chen et al., 2006; Starostik et al., 2002), we aimed to test their possible post-transcriptional regulation by miR-34a in the DLBCL cell lines introduced earlier. Quantitative RT-PCR of FoxP1 and Bcl6 expression after nucleoporation of U2932 and SUDHL4 cells with pre-miR-34a revealed that FoxP1, but not Bcl6, is a likely direct target of this miRNA (Figure 3A and data not shown). Protein levels of FoxP1 were also strongly reduced upon introduction of miR-34a into U2932 cells (Figure 3B). In order to measure a direct effect of miR-34a binding to its seed regions in the *foxp1* gene, we cloned the wild type sequence of the seed region, or a mutant version in which four of the six positions had been mutated, downstream of a luciferase reporter gene. Co-transfection of pre-miR-34a with the luciferase expression vector harboring the wild type seed region, but not the mutant version, blocked reporter gene expression as assessed by luciferase activity assay (Figure 3C). Interestingly, siRNA-mediated knock-down of Myc was as efficient as pre-miR-34a introduction in inhibiting FoxP1 expression (suppl. Figure 1). In summary, our results are in line with a recent report showing that miR-34a directly regulates FoxP1 expression by binding to at least one of its two predicted seed regions in the FoxP1 3'UTR (Rao et al 2010), and demonstrate that FoxP1 is a target of miR-34a and (an indirect target) of Myc in DLBCL cell lines. Our data further extend a previous report linking high FoxP1 expression to poor clinical outcome and a high risk of malignant transformation in MALT lymphoma patients (Sagaert et al., 2006).

The miR-34a target FoxP1 is a bona fide oncoprotein in DLBCL

As FoxP1 is a direct target of miR-34a, we asked whether FoxP1 is differentially expressed in gDLBCL and low grade MALT lymphoma. Indeed, a majority of gDLBCL, but very few of the low grade lymphoma cases spotted onto our tissue microarray showed reactivity with a FoxP1-specific antibody (Figure 4A,B). Interestingly, all FoxP1-positive cases also expressed Myc, irrespective of whether they were classified as low grade or high grade lymphomas. On the other hand, FoxP1 expression did not overlap with expression of

Bcl6, which is often used to distinguish between ‘GC’ and ‘ABC’ type DLBCL: similar proportions of FoxP1-positive cases were Bcl6-positive and –negative (data not shown). We postulated that the siRNA-mediated knock down of FoxP1 should have similar anti-proliferative effects in DLBCL cell lines as the delivery of miR-34a. This was indeed the case: knock down of FoxP1 by ~64% (suppl. Figure 2) blocked the proliferation of four ‘GC’ and ‘ABC’ type DLBCL cell lines at similar levels, and roughly as efficiently as the reintroduction of miR-34a (Figure 4C-F). In conclusion, our results suggest that the transcription factor FoxP1 is a direct target of miR-34a that is overexpressed in a majority of gDLBCL, but not in low grade lymphoma cases, and represents a bona fide oncoprotein in this disease entity.

Our results thus establish a mechanistic link between over-expression of Myc, the concomitant repression of Myc-regulated miRNA genes, and the deregulation of FoxP1 (see schematic in Figure 5). Chromosomal translocations involving the *MYC* locus are reported in the literature for 50-60% of gDLBCL, but are never detected in gastric low grade lymphomas and rarely found in nodal DLBCL (Kramer et al., 1998; van Krieken et al., 1990). While chromosomal translocations involving the *MYC* locus are the likely cause for Myc overexpression in gDLBCL, other alternative mechanisms of Myc deregulation are conceivable. Myc is itself known to be post-transcriptionally regulated by miRNAs. Interestingly, several Myc-regulating miRNAs (including miR-34a and let7) are also targets of Myc repression, establishing a feedback loop that aggravates and perpetuates the effects of Myc overexpression and may thus contribute to cancer progression. Several lines of evidence argue that miR-34a is a strong tumor suppressor miRNA in solid cancers. It is located on chromosome 1p36.22 in a region that has previously been associated with various malignancies, including lung cancer (Calin et al., 2004b); it is transcriptionally induced by the tumor suppressor p53 and its overexpression inhibits growth of various cancer types *in vitro* (He et al., 2007). The first proof of principle for the success of miR-34a “replacement therapy” in cancer treatment was recently reported using a preclinical model of non-small cell lung cancer; the local and systemic delivery of chemically synthesized miR-34a was achieved in this model by formulation with a lipid-based delivery reagent (Wiggins et al., 2010).

We demonstrate here for the first time that miR-34a has tumor suppressive properties in a hematopoietic malignancy. It is down-regulated in the malignant compared to the benign form of gastric lymphoma and its expression is directly regulated by Myc, which we show here to possess oncogenic properties in various DLBCL cell lines. Ectopic miR-34a re-expression prevents DLBCL growth. Finally, we demonstrate that miR-34a targets a suspected hematopoietic oncogene in gastric lymphoma, FoxP1, which is over-expressed in the malignant form of gastric lymphoma and which possesses bona fide oncogenic properties in DLBCL. Our results confirm and extend previous reports describing FoxP1 overexpression as a negative prognostic marker in gastric lymphoma and in nodal DLBCL (Hoeller et al; Sagaert et al., 2006). The post-transcriptional regulation of FoxP1 by miR-34a further provides a plausible explanation for the conundrum that FoxP1 is highly expressed in many lymphomas not harboring the rare chromosomal translocation t(3;14)(p13;q32), which juxtaposes the *FOXP1* and *IGH* gene loci in certain non-gastric MALT lymphomas and extranodal DLBCL (Haralambieva et al., 2006; Streubel et al., 2005).

In conclusion, we postulate here that miR-34 replacement therapy should be considered not only for the treatment of solid cancers, but may also prove beneficial in patients with miR-34a-negative, FoxP1-overexpressing hematopoietic malignancies such as gastric DLBCL. The beneficial therapeutic effects of miR-34a are anticipated due to the strong tumor suppressive properties of this miRNA, which are exerted via its oncogene target FoxP1.

Materials and Methods

Patient material and DLBCL cell lines

For miRNA expression analysis of archived patient material, consecutive cases of *H. pylori*-positive gastritis, of *H. pylori*-positive gastric low grade MALT lymphoma, and of gastric high grade MALT lymphoma were drawn from the surgical pathology files of the Institute of Pathology at the Cantonal Hospital St. Gallen, Switzerland. All data were blinded to guarantee patients' protection. All procedures were in agreement with the guidelines for use of human material in research issued by the Hospital's Ethics Committee. The DLBCL cell lines used were: two GC type (SUDHL4 and SUDHL6), one ABC type (U2932) and one unclassified DLBCL cell line (SUDHL7). All cell lines were maintained at 37°C in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum and antibiotics.

RNA extraction, microRNA expression profiling by microarray analysis and locked nucleic acid real time PCR for microRNA quantification

Total RNA was extracted from three 20 µm slices per sample of formalin fixed, paraffin embedded material using the RecoverAll total RNA Isolation kit (Ambion, Streetsville, Canada). Total RNA concentrations were measured using an ND-1000 spectrophotometer (NanoDrop Technologies). RNA integrity was confirmed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All microarray experiments were performed using the Agilent Human miRNA Microarray Kit version 10.0. 100 ng total RNA were hybridized per sample and processed according to the manufacturer's instructions. The arrays were scanned with an Agilent Technology G2565B scanner. The scanned images were gridded and analyzed using Agilent Feature Extraction Software version 9.5. The 795 human mature miRNAs included on the array were represented by 2421 probes; the average intensity of all probes corresponding to one miRNA was calculated. Normalization was performed using quantile normalization implemented in the bioconductor package PreprocessCore. The false discovery rate was computed using the Benjamini-Hochberg algorithm. All raw data of the microarray experiments are publicly accessible (http://www.ncbi.nlm.nih.gov/geo/). The expression of mature miRNAs was analysed using the miRCURY locked nucleic acid (LNA) microRNA PCR system following the

manufacturer's instructions (Exiqon, Vedbaek Denmark). Briefly, 10 ng of total RNA was subjected to cDNA synthesis using either miRNA- or U6 snRNA-specific primers. The cDNA template was diluted 1:10 and real time PCR reactions were performed following the manufactures' recommendations (LightCycler; Roche, Basel, CH). Calculations of miRNA expression levels were performed using the comparative $\Delta\Delta C_t$ method and normalized against U6 snRNA levels.

Immunohistochemical staining and Western blotting

The gastric lymphoma tissue microarray used in this study was constructed as described (Bernasconi et al., 2008) and included a total of 76 specimens, comprising 39 cases of gastric low grade MALT lymphomas and 37 cases of gastric DLBCL. The following primary antibodies were used: anti-MYC (N-262; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-FOXP1 (ICI2; Abcam, Cambridge, MA, USA). MYC and FOXP1 levels were assessed by counting the number of positively staining tumour cells and graded using the following expression scale: a negligible level of staining of 0-10% was recorded as negative, while low expression was between 10-60% and high expression was recorded when 60-100% tumour cells stained positive. Protein extracts were made using 2x Laemmli sample buffer (4% SDS, 20% glycerol, 120mM Tris [pH6.8]). Proteins were separated by SDS/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were probed with antibodies against FOXP1 (JC12; Abcam) or against α-tubulin (Santa Cruz Biotechnology) to control for equivalent gel loading.

Cell lines, transfections and luciferase reporter assays

On-target plus smartpool siRNAs for MYC, FOXP1 and a scrambled negative control was purchased from Dharmacon (Thermo Scientific, Lafayette, CO, USA). Precursor microRNA oligonucleotides (pre-miR-let-7a, pre-miR-34a, pre-miR-23a, pre-miR-26a, pre-miR-150 and pre-miR-15) and scrambled negative control oligonucleotides were purchased from Ambion. For the purpose of siRNA or miRNA inrtoduction into DLBCL cells, $1x10^6$ cells were nucleoporated using an Amaxa Nucleoporator (Gaithersburg, MD, USA) with the specified amount of pre-miR miRNA precursor or siRNA. After 48 h, cells were harvested for RNA and protein analysis. After 72 h, tumor cell proliferation was quantified by [^{3}H]

thymidine incorporation assay as previously described (Craig et al., 2010b). The pmirGLO Dual-Luciferase miRNA Target Expression Vector was purchased from Promega (Madison, WI, USA). HEK293T cells were seeded into 21-well plates at 1 x 10^5 cells/well 24 h before transfection. 1μg reporter plasmid containing the FOXP1 3'UTR or its mutants and 30nM mir-34a precursor molecules were contransfected into each well using the Fugene 6 transfection reagent (Roche) in triplicates. Luciferase assays were performed 24 hr after transfection using the Dual-Luciferase Reporter Assay System (Promega) with a Spectramax M5 reader (Molecular Devices, Sunnyvale, CA, USA).

Online supplemental material

Two supplemental tables listing the differentially expressed miRNAs between gastritis and low grade lymphoma samples (suppl. Table 1) and between low grade and high grade lymphoma samples (suppl. Table 2) are available with the online version of this manuscript. (Two supplemental figures and the corresponding legends are included in the Figure legends and Figures sections of this manuscript.)

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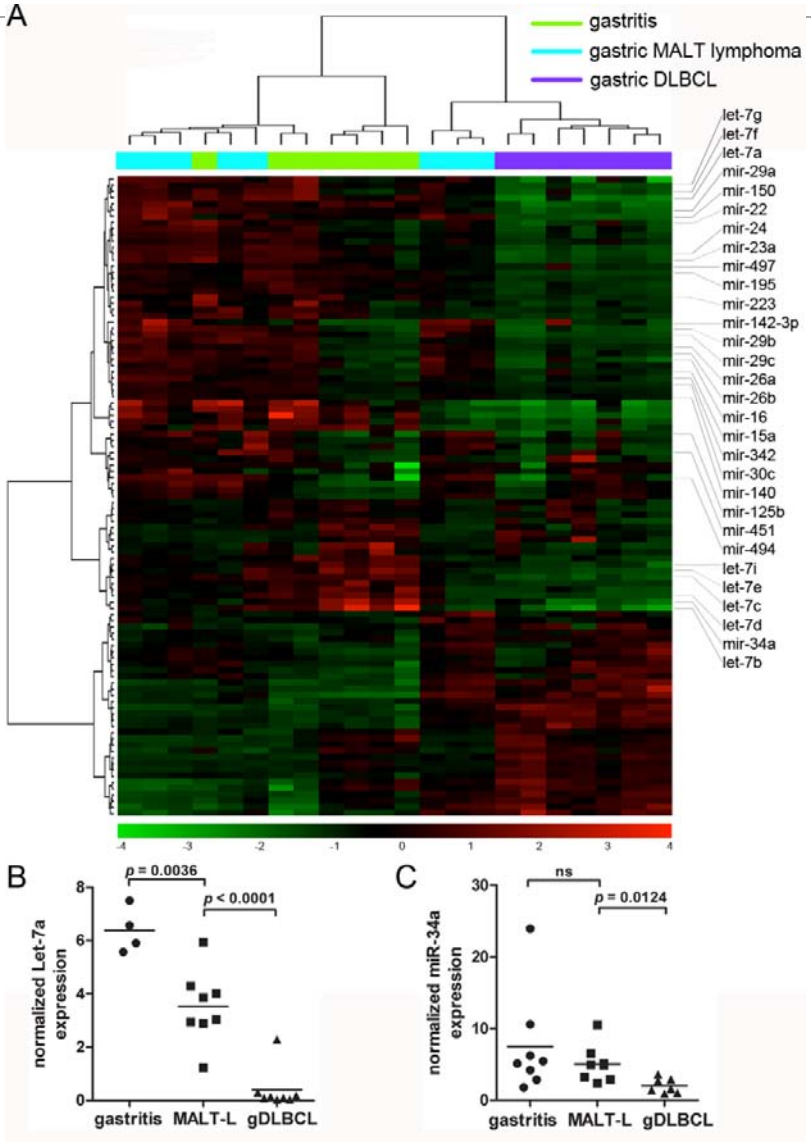


Figure 1. miRNA expression signatures of gastritis, low grade MALT lymphoma and gDLBCL. (A) The expression of 795 human mature miRNAs included in version 10.0 of the miRBase database was analyzed for seven cases of gastritis, eight cases of low grade MALT lymphoma and seven gDLBCL cases using microarray technology. The result of unsupervised hierarchical clustering of all miRNAs that varied across the 22 arrays with a standard deviation of $\log_2$ expression >0.05 is shown in the heat diagram. All known Myc-repressed miRNAs with lower expression in gDLBCL compared to low grade MALT lymphoma are annotated. (B,B) Quantification of let-7a (B) and miR-34a (C) expression in the samples shown in A by LNA real-time RT-PCR; absolute expression was normalized to U6 snRNA.

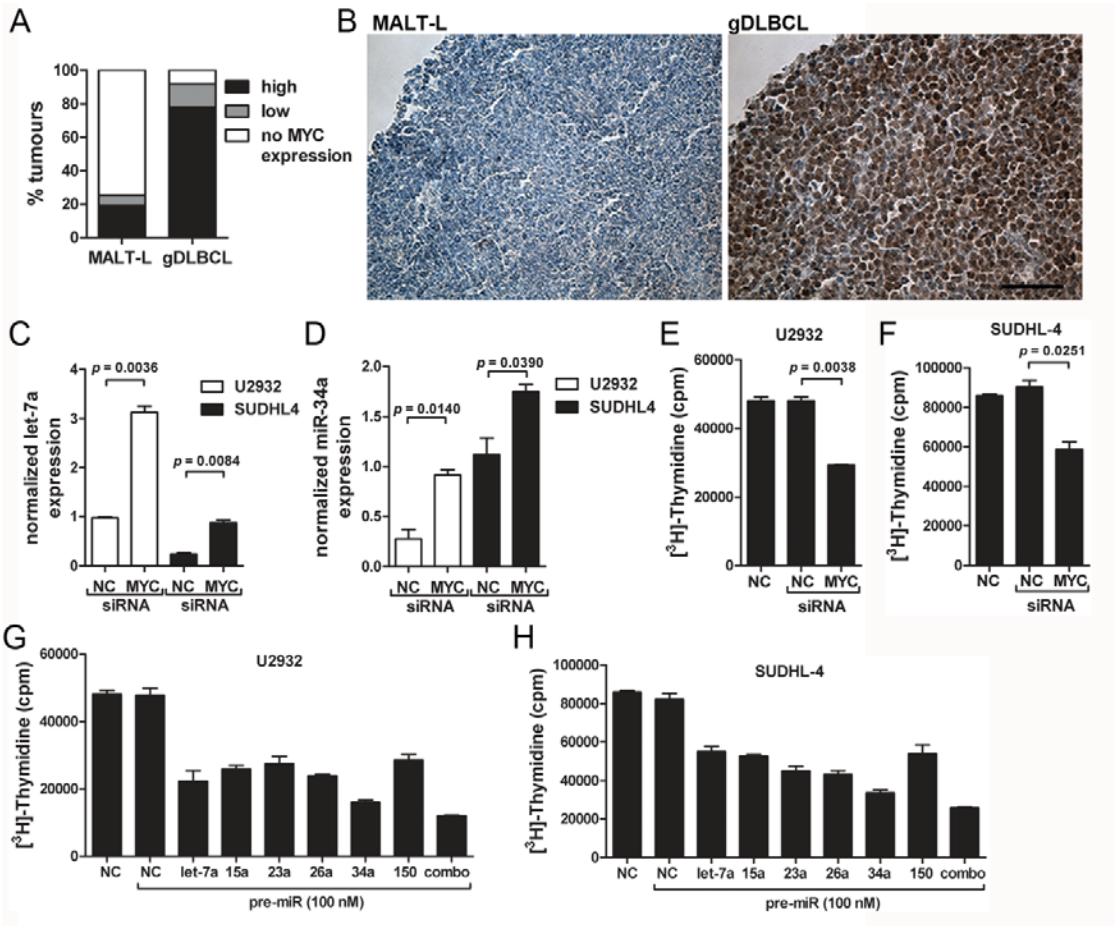


Figure 2. Myc is over-expressed in gastric diffuse large B-cell lymphoma (gDLBCL) and exhibits oncogenic properties in DLBCL cell lines *in vitro*. (A,B) Myc expression was analyzed by immunohistochemistry on a tissue microarray comprising 37 gDLBCL and 39 low grade MALT lymphoma (MALT-L) cases. The fraction of MALT lymphoma and gDLBCL cases with high, low and no Myc expression is indicated in A; representative micrographs are shown in B. The scale bar indicates 50μm. (C,D) Quantification of let-7a (C) and miR-34a (C) expression as determined by LNA real-time RT-PCR for the indicated cells lines (U2932, SUDHL4) 48h after electroporation with Myc-specific or scrambled (NC) siRNA. Expression values were normalized to U6 snRNA levels. (E,F) Proliferation as assessed by [^{3}H] thymidine incorporation of U2932 (E) and SUDHL-4 (F) cells 72 h after electroporation with Myc-specific or scrambled (NC) siRNA. (G,H) U2932 (G) and SUDHL-4 (H) cells were electroporated with the indicated pre-miRs or scrambled negative control (NC) oligonucleotide 72 h prior to the quantification of proliferation by [^{3}H] thymidine incorporation.

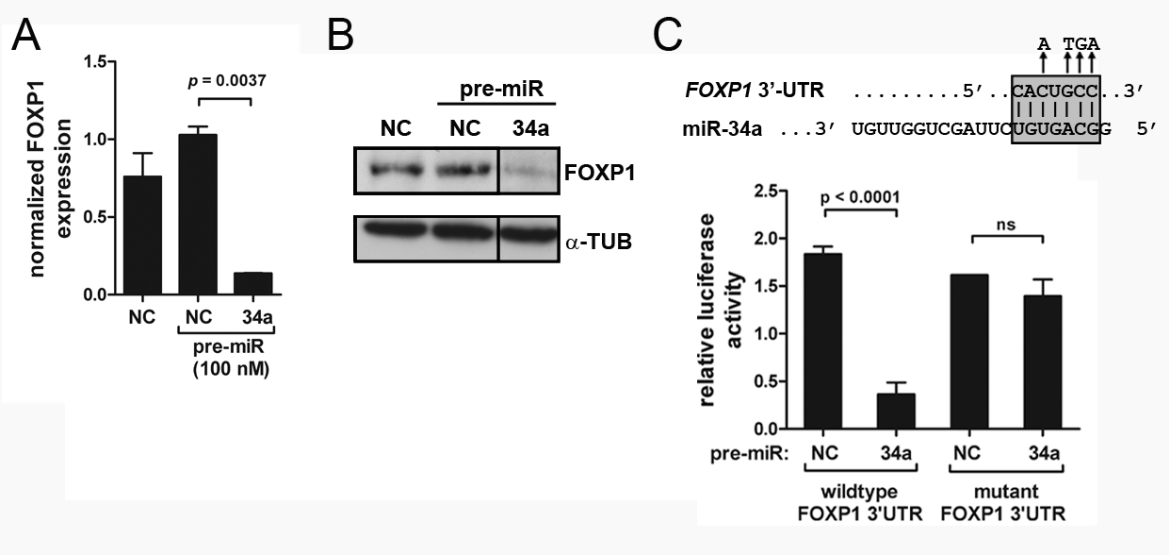


Figure 3. miR-34a directly targets FOXP1 in DLBCL. (A) U2932 cells were electroporated with pre-miR34a or a scrambled negative control pre-miR and analyzed with respect to FOXP1 expression 48 h later. FOXP1 transcript levels were normalized to GAPDH expression. (B) FoxP1 protein levels of the experiment described in A as analyzed by Western blot. α-tubulin levels are shown to control for equal loading. (C) Dual luciferase assay of HEK293T cells co-transfected with firefly luciferase constructs containing the wild-type or mutant miR-34a (arrows represent mutations) target site of the FOXP1 3'UTR region downstream of the luciferase reporter. Cells were co-transfected with either pre-miR-34a or a negative control scrambled oligonucleotide and the respective luciferase construct. Data are represented as relative luciferase activity.

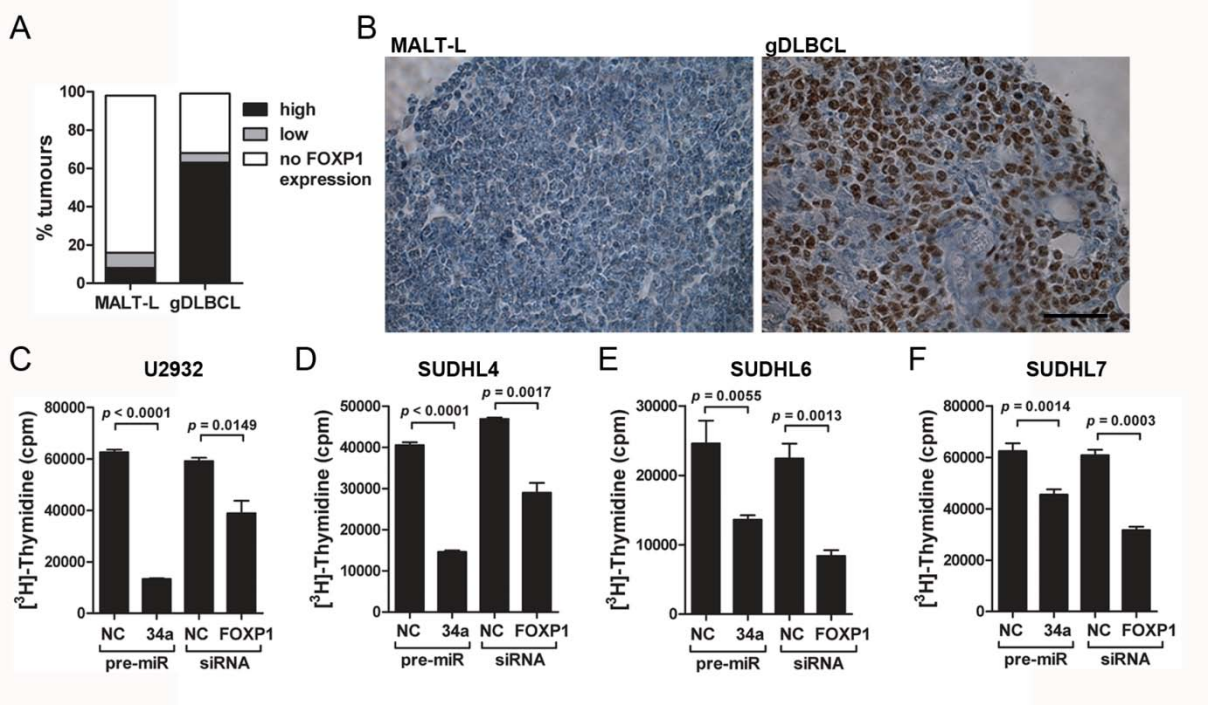


Figure 4. FOXP1 is a bone fide oncoprotein in DLBCL. (A,B) FoxP1 expression was analyzed by immunohistochemistry on the tissue microarray described in Figure 2. The fraction of MALT lymphoma and gDLBCL cases with high, low and no Myc expression is indicated in A; representative micrographs are shown in B. The scale bar indicates 50μm. (C-F) U2932 (C), SUDHL4 (D), SUDHL6 (E) and SUDHL7 (F) cells were electroporated with pre-miR34a, a FoxP1-specific siRNA or the respective negative control (NC) scrambled oligonucleotides 72 h prior to the quantification of proliferation by [^{3}H] thymidine incorporation.

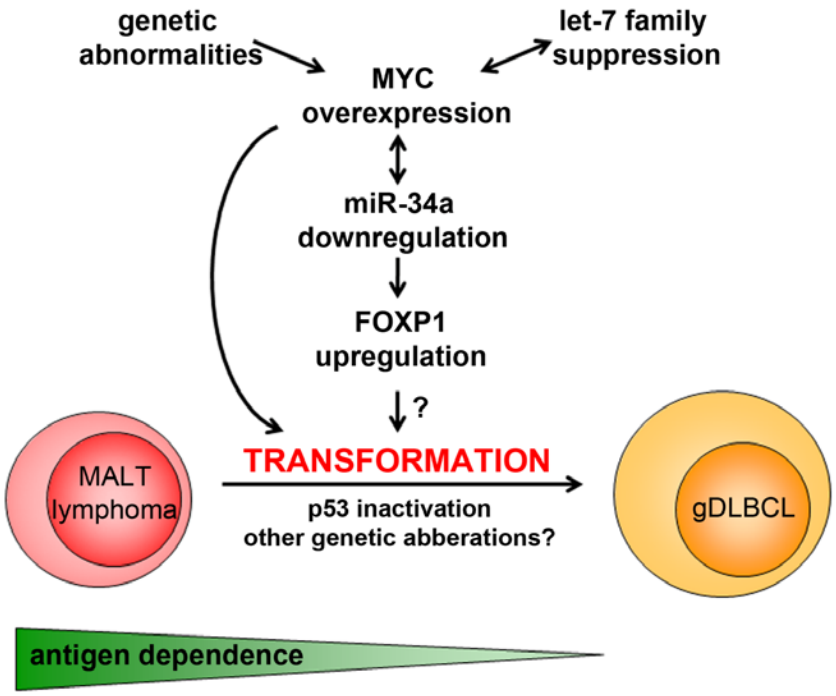
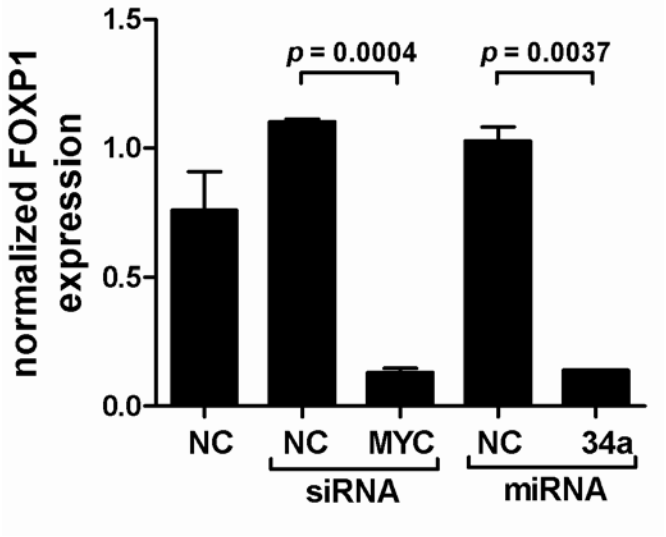
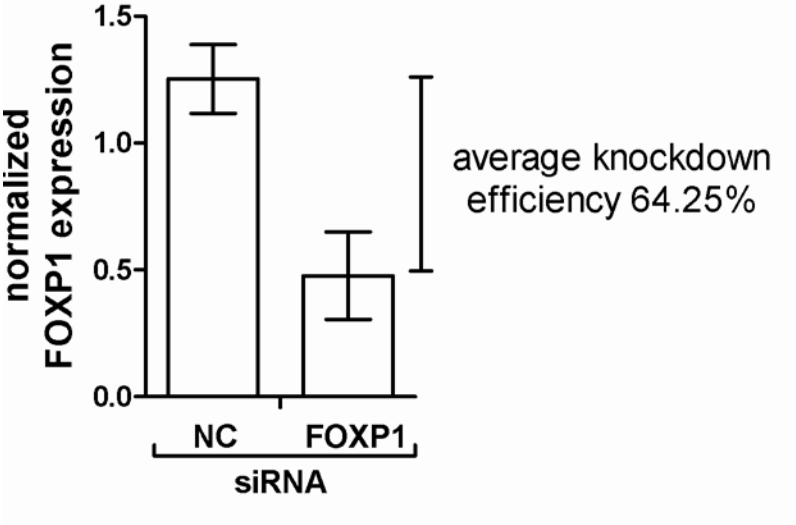


Figure 5. Schematic representation of the interplay of Myc, miR-34a and FoxP1 during malignant transformation of low grade gastric MALT lymphoma to gDLBCL. Myc overexpression can be caused by chromosomal translocations involving *Myc* and *Ig* loci or other genetic abnormalities, or by post-transcriptional regulation through miRNAs such as members of the let7 family. Antigen dependence is lost during malignant transformation.



Suppl. Figure 1. The knock-down of Myc inhibits FoxP1 expression as efficiently as miR-34a introduction. U2932 cells were electroporated with either a Myc-specific siRNA, pre-miR34a or a scrambled negative control siRNA or pre-miR and analyzed with respect to FOXP1 expression 48 h later. FOXP1 transcript levels were normalized to GAPDH expression.



Suppl. Figure 2. siRNA-mediated FoxP1 knock-down reduced FoxP1 expression by 64% on average. U2932, SUDHL4, SUDHL6 and SUDHL7 cells were electroporated with a FoxP1-specific siRNA or the respective negative control (NC) scrambled oligonucleotides 72 h prior to the quantification of FoxP1 expression.

Suppl. Table 1: List of differentially expressed miRNA genes between gastritis and low grade lymphoma samples with a *p*-value <0.05 and a fold-change >1.5.

***p*-value:** significance of differential expression between gastritis and MALT lymphoma samples as computed by t-test.

Fold-change: absolute fold change calculated as the ratio of miRNA signal intensities in MALT lymphoma vs. gastritis.

Significant differences between MALT lymphoma and gastritis

Upregulated miRNA	P-value	Fold change	Downregulated miRNA	P-value	Fold change
miR-1224-5p	0.025	2.318	let-7a	0.032	1.866
miR-135a*	0.028	1.774	let-7f	0.034	1.825
miR-150	0.006	2.758	miR-103	0.048	1.516
miR-150*	0.015	1.701	miR-107	0.010	1.562
miR-188-5p	0.049	2.668	miR-141	0.016	1.726
miR-345	0.039	1.757	miR-146a	0.021	2.408
miR-371-5p	0.044	1.65	miR-200a	0.026	1.604
miR-513b	0.035	1.594	miR-200b	0.044	4.403
miR-513c	0.005	1.637	miR-200c	0.028	1.823
			miR-21	0.021	1.912
			miR-223	0.020	2.369
			miR-31	0.044	1.550
			miR-34a	0.041	1.627
			miR-375	0.040	2.927
			miR-429	0.006	1.554
			miR-7	0.012	1.905

Suppl. Table 2: List of differentially expressed miRNA genes between low grade and high grade lymphoma samples with a *p*-value <0.05 and a fold-change >1.5.

***p*-value:** significance of differential expression between MALT lymphoma and high grade lymphoma samples as computed by t-test.

Fold-change: absolute fold change calculated as the ratio of miRNA signal intensities in MALTL vs. high grade lymphoma.

Significant differences between low-grade and transformed MALT lymphoma

Upregulated miRNA	P-value	Fold change	Downregulated miRNA	P-value	Fold change	Downregulated miRNA	P-value	Fold change
miR-1224-5p	0.024	1.988	let-7a	<0.001	7.097	miR-200b	0.040	4.228
miR-1225-3p	0.000	1.615	let-7b	0.003	4.259	miR-200c	0.048	4.137
miR-1225-5p	0.002	3.55	let-7c	0.001	2.677	miR-21	0.027	3.241
miR-1226*	0.000	1.88	let-7d	0.005	2.139	miR-214	0.002	1.496
miR-1228	0.000	2.461	let-7e	0.003	2.317	miR-22	0.036	2.400
miR-125a-3p	0.001	2.405	let-7f	<0.001	5.447	miR-223	<0.001	2.095
miR-125b-1*	0.021	1.933	let-7g	<0.001	5.426	miR-23a	<0.001	3.880
miR-134	0.001	3.124	let-7i	0.002	2.344	miR-23b	<0.001	4.277
miR-135a*	0.026	1.598	miR-100	0.024	1.681	miR-24	<0.001	3.758
miR-149*	0.001	1.699	miR-103	0.001	2.018	miR-26a	<0.001	3.317
miR-150*	0.000	2.895	miR-107	<0.001	2.161	miR-26b	<0.001	2.908
miR-187*	0.004	1.502	miR-10a	0.024	1.593	miR-27a	0.002	3.259
miR-188-5p	0.013	3.319	miR-125b	0.031	2.424	miR-27b	0.001	3.026
miR-296-5p	0.000	2.246	miR-126	0.008	1.905	miR-28-5p	0.003	1.556
miR-320a	0.001	1.452	miR-130a	<0.001	1.827	miR-29a	<0.001	6.410
miR-371-5p	0.000	3.386	miR-140-3p	0.001	1.719	miR-29b	<0.001	4.766
miR-373*	0.000	2.167	miR-142-3p	0.006	4.168	miR-29c	<0.001	4.425
miR-483-5p	0.009	1.909	miR-143	0.047	1.780	miR-30a	0.009	1.767
miR-516a-5p	0.013	1.52	miR-145	0.029	2.916	miR-30b	0.001	3.081
miR-557	0.000	2.328	miR-146a	0.027	1.829	miR-30c	0.001	2.127
miR-572	0.017	3.188	miR-150	<0.001	8.726	miR-30e	0.002	1.597
miR-575	0.005	1.838	miR-151-5p	0.001	1.924	miR-338-3p	0.008	1.536
miR-601	0.002	1.782	miR-15a	<0.001	2.431	miR-342-3p	0.001	2.269
miR-623	0.003	1.624	miR-15b	0.013	1.947	miR-34a	0.002	2.298
miR-630	0.010	3.211	miR-16	<0.001	4.679	miR-497	<0.001	2.002
miR-638	0.027	4.148	miR-192	0.019	3.761	miR-768-3p	<0.001	5.400
miR-659	0.003	1.752	miR-194	0.044	2.361			
miR-663	0.000	2.321	miR-195	<0.001	2.815			
miR-671-5p	0.020	2.9	miR-199a-3p	<0.001	2.327			
miR-939	0.010	2.616	miR-199a-5p	0.002	1.669			
miR-99b*	0.001	1.536	miR-200a	0.033	2.088			

4 DISCUSSION AND OUTLOOK

MALT lymphoma attracts much attention owing to its unique clinical and molecular features and represents a paradigm for the study of inflammation-induced lymphomas. Despite keen interest in this disease, the molecular pathogenesis of MALT lymphoma has remained poorly understood. Research has been hindered by several limitations such as, the lack of an immortalised cell line in conjunction with the customary challenges involved in obtaining human patient material. The availability of a murine MALT lymphoma model coupled with our fortunate acquisition of rare human material gave us a unique standpoint to address several key unresolved issues concerning the molecular detail of MALT lymphomagenesis. This partly entailed revisiting familiar questions, namely concerning the role of infiltrating T cells and antigen-recognition, while on the on other hand, novel subject areas such as miRNA deregulation in tumourigenesis were also explored. The following sections discuss the main outcomes and impact of our research with a special focus on relevant future research prospects.

4.1 Promiscuity is a pathogenic feature of MALT lymphoma

Although multiple studies on MALT lymphoma have alluded to a role for antigen in the pathogenesis of this disease, the question has remained whether this interaction occurs directly via the surface Ig of the tumour B cells and if so, what are the exact ligands involved. Previously, the Ig of certain MALT lymphomas have been found to have a rheumatoid factor reactivity[66] and binding of tumour Ig to various structures of normal human tissues (follicular dendritic cells, venules, epithelial cells, and connective tissue) has also been described.[75, 172] Our research on this ongoing theme of antigen specificity revealed that the tumour B cells behave in a polyspecific manner; not only did the recombinant MALT lymphoma-derived Igs exhibit broad reactivity against a panel of diverse antigens in a series of enzyme immunoassays, but we were further able to demonstrate in an *ex vivo* setting that murine tumour cells proliferate in response to the same panel of cognate antigens. Our experimental finding of polyreactivity was further supported by molecular analysis of MALT lymphoma

Igs, wherein many of the known hallmarks of polyreactive antibodies such as IgM class, a longer than average CDR3 length and restricted V_H gene usage were detected.

Overall, our results suggest that the development of MALT lymphoma may be facilitated by an array of local self- and foreign antigens, providing direct antigenic stimulation of the tumour cells via their BCR. In a broad sense, our findings provide experimental support for the expanding ideology that antigen-stimulation via the BCR is an important pathogenic feature of many B-cell lymphomas, such as follicular lymphoma[173-175]and Burkitt's lymphoma.[174, 176-178] The phenomenon of polyreactivity is a well known concept among B cells, with a major proportion of the natural antibody repertoire consisting of polyspecific antibodies.[179] Despite their preponderance, little experimental data exists concerning their biological function; however, it is currently assumed that they may play a role in the development and maintenance of immunological tolerance. The general mechanism to explain multispecific ligand recognition by antibodies is that the antigen-binding pocket possesses a degree of conformational flexibility thereby, permitting the receptor to engage multiple ligands.[179, 180]

In connection to other B-cell malignancies, polyreactivity is not a unique feature of MALT lymphoma. Chronic lymphocytic leukaemia (CLL) is the most prevalent form of adult leukaemia in the Western world[181] and like MALT lymphoma, it is considered to be an antigen-driven neoplasm. Several studies have noted that tumour cells with a polyreactive binding capacity distinguish CLL cases with a worse clinical prognosis from those with better outcomes.[182-184] In contrast to MALT lymphoma, most polyreactive CLL cells have not undergone somatic hypermutation, rather *IgV* sequence analysis suggests that the CLL clones have undergone various types of receptor editing.[185-187] In receptor editing, antigen encounter induces autoreactive B cells to undergo secondary rearrangements of the light chain to augment their specificity from self to non-self.

The persistence of polyreactivity, particularly in the face of receptor configuration, is somewhat paradoxical since the immune system is ostensibly programmed to delete such inappropriate and deleterious antigen reactivity. Remarkably, we found that the only

recombinant MALT lymphoma-derived Ig that failed to exhibit polyreactivity was also the only unmutated antibody among those analysed. Of future interest would be to determine if the link between mutation status and polyreactive binding profile remains consistent in larger studies that include more of the rare unmutated cases. One way we have began to elucidate the role of somatic hypermutation in MALT lymphoma development is by experimental infection of activation-induced cytidine deaminase (AID)-knock out mice with *H. felis*. The enzyme, AID, is known to be essential to both somatic hypermutation and class switch recombination of Ig[188, 189] and is highly expressed in a variety of B-cell neoplasms, including MALT lymphoma.[190-193] We observed that only one out of the seven infected $AID^{-/-}$ mice actually developed lymphomas after 18 months of infection, while the remaining animals were tumour-free (data not shown). These preliminary data therefore support the notion that AID-mediated antigen-receptor gene modification processes may be a principal contributor to the pathogenesis of MALT lymphoma.

In light of the simultaneous occurrence of polyreactivity and receptor configuration, a recent investigation demonstrated that pathogenic polyreactive antibodies have undergone a higher rate of somatic hypermutation compared with pathogenic monoreactive autoantibodies.[194] Furthermore, many investigators have now demonstrated that lupus-inducing autoantibodies tend to be polyreactive or cross-reactive rather than monospecific.[195-197] These results support the recent paradigm shift that polyreactive autoantibodies are more pathogenic than monoreactive autoantibodies. In this context, polyreactivity certainly lends well to the hypothesis that B cells with a promiscuous antigen receptor have a higher risk of accruing genetic lesions and undergoing subsequent malignant transformation due to the sheer availability of cognate antigen that may trigger rapid cell divisions. It is therefore tempting to speculate that a polyreactive BCR may provide an important growth advantage for the neoplastic B-cell clone at an early stage; as transforming events accumulate, dependence on the presence of the BCR may be lost symbolising the evolution of MALT lymphoma into aggressive gDLBCL. The fact that some gDLBCLs have regressed upon eradication of *H. pylori*, suggests that these lymphomas may have preserved their antigen dependence and thus lures us to consider the BCR as an integral pathogenic feature of these lymphomas. Further research is required to determine the proportion of

gDLBCLs that are sensitive to eradication therapy and whether these tumour cells behave in a polyspecific manner.

4.2 Tumour-infiltrating T cells are essential for MALT lymphoma

The discovery of a large infiltrating $CD4^+$ T-cell population in the MALT lymphoma environment triggered an exciting cascade of research aimed at characterising and understanding the significance of this populace (see section 1.1.4.2). Tumour-infiltrating $CD4^+$ T cells are known to co-express CD40L and the activation marker CD69 and strongly produce Th2 cytokines such as IL4.[11, 198] Several groups have reported that tumour-infiltrating T cells are specific for *H. pylori*, and have postulated that T cells rather than tumour B cells constitute the *Helicobacter*-specific component of the tumour mass.[32, 199] Finally, an important finding by Greiner et al.[63] was that MALT lymphoma cells are stimulated to proliferate in the 'CD40 culture system', that is, under constant stimulation with an agonistic CD40 antibody in conjunction with Th2 cytokines. This observation led to the conclusion that CD40 signalling was essential for tumour B-cell proliferation.[63]

We set out to further examine the influence of T cells in the evolution of MALT lymphoma. To this end we established transient *ex vivo* cultures of murine MALT lymphoma cells and found, using this model system, that systemic depletion of all $CD4^+$ or $CD40L^+$ cells from the cultures largely abrogated tumour cell proliferation; the depletion of $CD25^+$ T cells had a slightly weaker, but nevertheless significant effect. We conclude that these T cells are essential for providing B-cell stimulatory signals. Unexpectedly however, blocking the direct interaction between CD40L and its receptor failed to quell tumour cell proliferation. Our data therefore suggests that tumour cell proliferation is enhanced by the presence of intratumoural $CD4^+$ T cells in a CD40/CD40L-independent manner. Our data confirm previous results by Greiner et al.[200] who also reported CD40L expression on human MALT lymphoma infiltrating T cells. However, our results challenge the dogma that tumour B cells rely on direct contact with T cells via CD40 signalling. Although the tumour B cells can in principle respond to agonistic CD40 stimulation due to high CD40 expression, the direct

interaction between the B and T cells through CD40/CD40L appears not to be essential for tumour cell proliferation, at least in an *ex vivo* setting.

Our research lays a fertile groundwork for future efforts aimed at delineating the soluble activated T cell-derived factors or potential non-CD40-mediated contact dependent mechanisms driving the proliferation of the tumour cells. Prospective studies should foremost include the manipulation of the Th2 cytokine profile of the MALT lymphoma environment to identify the chief soluble tumour-promoting mediators. In regards to alternative contact dependent mechanisms, the CD28/CTLA4 ligand CD86 represents a key suspect worthy of investigation; CD86 is expressed on MALT lymphoma cells[58-60] and evidence has surfaced that this costimulatory molecule may promote T-cell mediated neoplastic B-cell growth.[201]

An unprecedented finding of our study was the high concentration of $FoxP3^+$ Treg cells within the tumour-infiltrating T-cell compartment, both in the murine tumours and in human patient biopsy material. Tregs are known to protect the host from autoimmune disease and mediate peripheral tolerance by suppressing autoreactive immune cells.[202] Moreover, it is becoming increasingly clear that Tregs are also involved in fostering an immune-privileged niche and blocking antitumour immunity.[203-205] Consistent with this concept, is the overrepresentation of Treg cells reported in various types of cancer.[203, 205-211] In our model of *Helicobacter* infection-induced MALT lymphoma, monoclonal antibody-mediated depletion of $CD25^+$ Treg cells reduced the tumour burden as efficiently as the depletion of all $CD4^+$ T cells, presumably by inducing the regression of pre-existing tumours. This finding is supported by the equally robust effect of Treg depletion on tumour cell proliferation observed *ex vivo*.

Several animal tumour models have demonstrated improved tumour immunity and tumour regression upon *in vivo* administration of a CD25-specific antibody.[212-215] Given that CD25-specific antibody treatment has been proven to efficiently deplete Treg cells, enhance tumour immunity and result in mouse tumour regression, reducing the Treg content of cancer patients may be of therapeutic value. However, conjecture surrounds the ability of CD25 depletion to specifically eliminate Tregs in the tumour microenvironment, since CD25 is also

expressed on the cell surface of activated T cells.[216] In spite of this, CD25 depletion has been shown not to result in elimination of effector T cells, but rather to significantly enhance activated T-cell numbers.[217] Furthermore, CD25-directed Treg depletion has proceeded to clinical application and shown to decrease the number of $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells while heightening antitumour T-cell responses as measured in the blood of patients with renal cell carcinoma and metastatic melanoma.[218, 219] Although depletion of Treg cells is an attractive option, careful clinical studies are needed with large patient populations to link Treg depletion with potential clinical efficacy.

The observation that Tregs are overrepresented in the MALT lymphoma environment raised the question of whether these cells were actively recruited to the tumour site. Our results provide evidence that the malignant B cells are involved in the chemotaxis of intratumoural Treg by secretion of two chemokines CCL17 (also known as thymus and activation-related chemokine, TARC) and CCL22. These chemokines, either alone or in combination, had previously been shown to recruit Tregs into epithelial cancers of the stomach[220] and ovaries.[205] Notably, Curiel et al.[205] demonstrated that an *in vivo* blockade of CCL22 significantly reduced Treg migration to the tumour site. These reports therefore suggest that in addition to depleting Tregs, targeting the molecules involved in Treg-cell tumour trafficking represents an alternative strategy for controlling Treg function.

Finally, we have shown that the intratumoural Tregs in MALT lymphoma specimens are highly suppressive against autologous effector T cells, more so than natural $CD25^+$ Tregs isolated from the corresponding spleens and mesenteric lymph nodes of the same mouse. Although our results collectively indicate an important role for Treg cells in the regulation of MALT lymphoma growth, it remains uncertain if the suppressive activity of intratumoural Tregs is required for their tumour-promoting function. In dispute of the notion that malignant B cells benefit from intratumoural Tregs through mechanisms of immune privilege, we alternatively propose the concept that low grade MALT lymphomas may be directly stimulated by Treg-derived contact-dependent or soluble signals. Our first line of evidence stems from the observation that Treg depletion inhibits tumour B-cell proliferation *in vitro*, an effect unlikely due to enhanced antitumour immune responses. Secondly, low grade

MALT lymphoma harbours more Tregs and overall infiltrating T cells than their transformed autonomously growing gDLBCL counterpart.

Our data is similar to previous findings in other non-Hodgkin's lymphoma, where an increased percentage of $FOXP3^+$ Treg cells is predictive of better prognoses.[221-223] In particular, Carreras et al.[221] reported a marked decrease in the number of Tregs during the transformation of follicular lymphoma to DLBCL, suggesting that these cells may play a role in tumour progression. The authors proposed that Tregs may facilitate lymphoma progression due to their inhibitory effects on the tumour B cells. This notion is supported by reports that Tregs are capable of directly suppressing B cells by inhibiting Ig production[224] or by inducing cell death mediated by a cytotoxic-dependent pathway.[225] By way of contrast, a high Treg frequency appears to closely correlate with poor prognoses and reduced survival rates for many other types of cancers.[205, 207-209] In this setting, high numbers of Tregs are thought to benefit the tumours by blocking antitumour immune responses, thereby fostering an immune privileged tumour microenvironment.

We conclude from our results that MALT lymphomas are infiltrated by Tregs that are highly suppressive towards effector T cells on one hand, and essential for optimal tumour B-cell proliferation on the other. Future research is warranted to elucidate the mechanisms through which Tregs promote tumour B-cell proliferation in the *ex vivo* culture model we have established; for example, the neutralisation of soluble or contact-dependent Treg-derived markers such as IL10, TGFβ and CTLA4 should clarify whether these signals are crucial for promoting tumour cell proliferation *ex vivo*. Taken together, we provide strong evidence that both antigen stimulation and infiltrating T cells may synergistically contribute to the development of MALT lymphoma during persistent *H. pylori* infection (Figure 4.1).

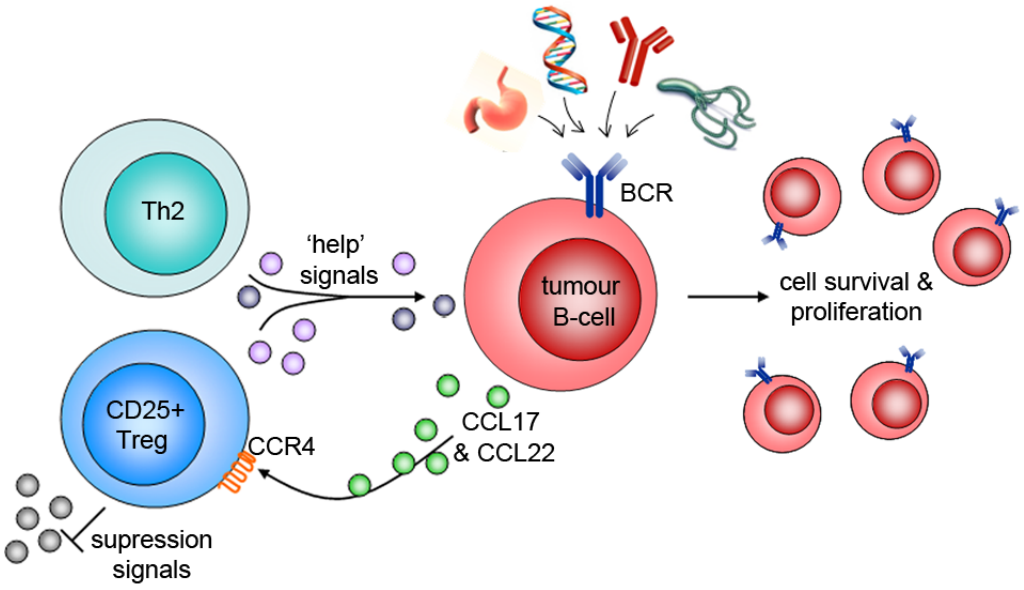


Figure 4.1 B-cell receptor signalling and CD40 ligand-independent T-cell help cooperate to drive MALT lymphoma cell proliferation. Tumour B cells proliferate in response to a variety of antigenic stimuli via a polyreactive B-cell receptor (BCR). In parallel, tumour-infiltrating T-helper type 2 (Th2) and functional regulatory T cells (Tregs) provide signals required for the proliferation and survival of MALT lymphoma B cells. The tumour cells actively recruit the tumour cells through secretion of the Treg-attracting chemokines CCL17 and CCL22 which bind to a shared receptor on the Treg surface, CCR4.

4.3 MicroRNA deregulation: a novel player in the development and progression of MALT lymphoma

4.3.1 miR-203 controls the oncogene ABL1 in MALT lymphoma with therapeutic consequences

A large body of evidence has documented the influence of miRNAs in tumourigenesis. However, the contribution of miRNA to the pathogenesis of MALT lymphoma has remained in the dark. To shed light on this subject, we used a genome-wide microarray approach to identify miRNAs potentially involved in the various stages of MALT lymphomagenesis. As expected, the miRNA expression signature of MALT lymphoma was more closely related to gastritis than to gDLBCL. This observation clearly reflects the biological similarities shared between the former two disease entities compared to the latter

more aggressive tumour counterpart. Nevertheless, the molecular events that drive the transition from reactive *H. pylori*-dependent gastritis to MALT lymphoma are poorly demarcated.

Our microarray-based miRNA transcriptome analysis identified *miR-203* as one of the most strongly downregulated miRNAs in gastric lymphoma. We verified that miR-203 was indeed significantly downregulated in MALT lymphoma compared to gastritis and normal tonsil tissue using real-time PCR. Silencing of the *miR-203* gene locus in both low and high grade MALT lymphoma was found to be due to extensive promoter CpG hypermethylation. Several lines of evidence support a tumour suppressive role for miR-203 in MALT lymphoma. Its expression is downregulated in cancers of the liver,[226] central nervous system,[227] esophagus,[228] and in certain types of leukaemia.[229] Experimental inhibition of miR-203 using an antisense approach was shown to enhance the growth of lung carcinoma cell lines.[230] Conversely, the restoration of miR-203 expression significantly reduced cellular proliferation of hepatocellular carcinoma,[231] types of leukaemia such as chronic myelogenous leukaemia (CML)[229] and head and neck squamous cell carcinoma.[232] Finally, an experimentally validated target of miR-203 is the ABL1 non-receptor tyrosine kinase proto-oncogene.[229]

The oncogenic role of ABL1 has been extensively characterised in human leukaemias that harbour the t(9;22) reciprocal translocation fusing the *BCR* and *ABL1* gene loci, known as the Philadelphia chromosome (Ph). This translocation is a hallmark of CML and is also present in a fraction of B-cell acute lymphoblastic leukaemias (ALL) that have a particularly poor prognosis.[233] Although MALT lymphomas are Ph-negative, we found that ABL1 is overexpressed in both human and murine MALT lymphoma samples in comparison to corresponding gastritis material. Our findings are in line with the recent observations that ABL1 is de-regulated in a variety of Ph-negative malignancies, such as CLL,[79, 234, 235] breast,[236] thyroid[237] and lung cancer.[238]

The ABL1 protein is ubiquitously expressed and localised both in the nucleus and cytoplasm where it is involved in diverse processes such as, the regulation of cell growth, survival, proliferation, migration and cellular responses to DNA damage and oxidative stress.[239] Although ABL1 is expressed in all mammalian tissues, its levels are highest in the thymus, spleen, and testes.[240] It is clear that ABL1 expression is crucial for normal B-cell development, as mice homozygous for a mutation in the *ABL1* gene have multiple defects including high postnatal mortality, thymic and splenic atrophy and a T- and B-cell lymphopenia.[240] ABL1 is thought to regulate B-cell proliferation downstream of the BCR, possibly by directly interacting with, and phosphorylating, the BCR co-receptor CD19.[241] B cells derived from *ABL1*$^{-/-}$ mice exhibit an impaired proliferative response following BCR engagement.[241] In addition, levels of ABL1 are elevated in the cytosol following BCR engagement in the B-cell lines Raji and Ramos.[241] Consistent with its involvement in BCR signalling, ABL1 is believed to play a pathogenic role in CLL cells through a mechanism involving constitutive NF-κB activation.[234] Aberrant NF-κB signalling is a hallmark of MALT lymphoma. Thus deregulated ABL1 expression points to a possible mechanism for uncontrolled NF-κB activation in MALT lymphoma. Despite these emergent clues, the precise oncogenic action of ABL1 in MALT lymphoma awaits confirmation and points to an exciting area of future research.

Given that ABL1 is implicated in the dysregulation of the NF-κB pathway via BCR signalling and is overexpressed in MALT lymphoma we hypothesised that ABL1 inhibition, either by *miR-203* re-expression or inhibition of ABL1's tyrosine kinase activity would block the *Helicobacter*-induced proliferation of primary MALT lymphoma cells. Indeed, both lentiviral delivery of miR-203 and treatment of explanted murine MALT lymphoma cells with the tyrosine kinase inhibitor imatinib efficiently prevented their proliferation. Administration of chronically *Helicobacter*-infected mice with imatinib, prevented MALT lymphomagenesis, suggesting that ABL1 inhibition may be a valid strategy for the treatment of patients that are refractory to eradication therapy. Indeed the therapeutic effect of imatinib in MALT lymphoma probably reflects the critical role of antigen-induced signalling from the BCR in the pathogenesis of the tumour.

As well as being the current first-line therapy for CML, imatinib has been approved for the treatment of chronic eosinophilic leukaemia, gastrointestinal stromal tumours and Ph-positive ALL (Ph-positive).[233, 242] In addition, pre-clinical studies recommend imatinib for the treatment of CLL patients overexpressing ABL1.[79, 234] Given our results, it is worthwhile to consider the potential benefit of imatinib treatment for gDLBCL which, in resemblance its low grade counterpart, is miR-203 deficient. However, the question remains open whether gDLBCLs also overexpress ABL1, thereby rendering the lymphoma sensitive to imatinib treatment. We therefore await the opportunity to treat ABL1-postive, miR-203-negative DLBCL cells with imatinib.

In conclusion, (as summarised in Figure 4.3) we propose that the evolution of *Helicobacter*-associated gastritis to low grade MALT lymphoma may be epigenetically regulated through hypermethylation of the *miR-203* promoter; the subsequent silencing of *miR-203* expression results in deregulation of ABL1, which in turn drives MALT lymphoma cell proliferation. Our results identify ABL1 as a promising new target for the treatment of MALT lymphoma; in particular, the pharmacological inhibition of this kinase may be of therapeutic benefit to patients who are refractory to antibiotic eradication therapy.

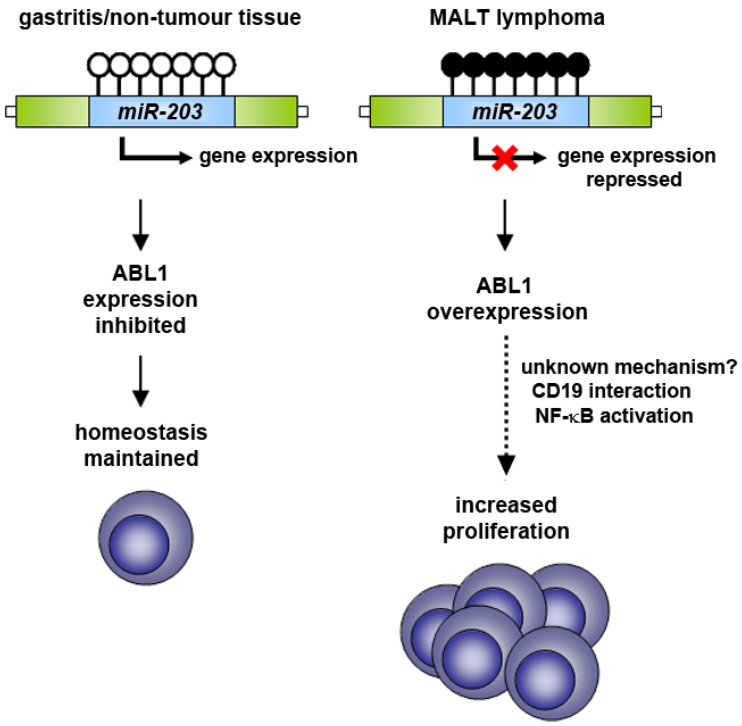


Figure 4.3 Model of ABL1 regulation by *miR-203* in MALT lymphoma. *MiR-203* is specifically epigenetically silenced in MALT lymphoma compared to non-tumour tissue. The *miR-203* promoter is embedded in a CpG island which remains unmethylated in gastritis but is hypermethylated in MALT lymphoma. The loss of *miR-203* leads to upregulation of ABL1 expression which contributes to uncontrolled proliferation of the neoplastic B cells. *Open* circles depict unmethylated CpG sites and the *filled* circles represent methylated CpG sites

4.3.2 MYC-mediated repression of miR-34a promotes high grade transformation of MALT lymphoma through its target FOXP1

To elucidate the biological mechanisms underlying the event of MALT lymphoma transformation into gDLBCL we compared the miRNome of low and high grade MALT lymphoma samples. Of the large number of miRNAs differentially expressed between these two disease entities, the vast majority were downregulated in the gDLBCL cases analysed. Global reduction of miRNA expression is a hallmark of cancer progression and is thought to contribute to neoplastic transformation by allowing an increased expression of oncoproteins.[164] Interestingly, the transcription factor MYC is known to directly suppress the expression of a large number of human miRNAs, thereby contributing to the phenomenon of reduced abundance of select miRNAs in cancer cells.[162, 163, 243] Moreover, many of these MYC-associated miRNAs have known tumour suppressive activity thereby strengthening the tumour promoting program of MYC.

Remarkably, the majority of known MYC-repressed miRNAs were found to be significantly downregulated in the transformed high grade, but not the low grade cases of MALT lymphoma analysed. This implies that MYC may dramatically influence the miRNA expression profile of gDLBCL. To test this hypothesis, we compared MYC expression levels between cases of low and high grade MALT lymphoma using a tissue microarray platform and demonstrated that aberrant MYC expression is strongly associated with gDLBCL. This correlation is not entirely surprising since the presence of *MYC* rearrangements are often detected in gDLBCL patients, while rarely being found in low grade MALT lymphoma and nodal DLBCL.[105, 107] An alternative source for MYC overexpression is based on evidence that MYC itself is post-transcriptionally regulated by certain MYC-repressed miRNAs thus establishing a feedback loop that exacerbates and strengthens the oncogenic effects of MYC.[162, 244] For instance, in Burkitt's lymphoma translocation of the *MYC* gene is generally considered a hallmark of this disease; however, a very small number of cases do not exhibit any *MYC* gene rearrangements, yet still overexpress MYC.[244] In these translocation-negative Burkitt's lymphoma cases, suppression of the miRNAs let-7c and miR-34b (which negatively regulate MYC) has been put forward as the probable cause of MYC overexpression.[244]

To assess the causal link between aberrant MYC expression and miRNA downregulation in gDLBCL, we transiently knocked down MYC by siRNA in DLBCL cell lines. Upon MYC silencing, we confirmed the concomitant upregulation of known MYC-repressed miRNAs, let-7 and miR-34a. In addition, the growth of the tumour cells was significantly blocked bolstering the notion that MYC expression drives DLBCL proliferation, possibly via downregulation of tumour suppressive miRNAs. To explore the potential tumour suppressive impact of MYC-repressed miRNAs in lymphomagenesis, we re-expressed a panel of these miRNAs in DLBCL cell lines. Although all six of the miRNAs that were examined significantly reduced the proliferation of the tumour cells, the restoration of miR-34a levels had the most potent effect on tumour cell proliferation. Ectopic expression of miR-34a has also been found to display an anti-proliferative phenotype in several solid tumour cell lines.[245-249] A recent breakthrough concerning miR-34a was the demonstration of lung tumour growth inhibition in mice following systemic delivery of miR-34a oligonucleotide mimics.[260] Enforced expression of miR-34a may therefore hold significant promise as a novel therapeutic modality for human gDLBCL.

In support of its role as a tumour suppressor, miR-34a has been shown to regulate genes involved in apoptosis, cell growth and cell-cycle regulation, including *MYCN*, *BCL2* and *CDK6*.[145, 250, 251] The anti-oncogenic nature of miR-34a is reinforced by the finding that it is transcriptionally induced by the TP53 tumour suppressor.[252] To further investigate the anti-tumourigenic action of miR-34a we and others have extended the list of its direct targets to include the forkhead transcription factor FOXP1.[253] In addition, we found using our tissue microarray platform that high FOXP1 expression strongly correlates with gDLBCL. Recent attention has focused on FOXP1 and its role in tumourigenesis due to its upregulation in a variety of B-cell neoplasias. In particular, Sagaert et al.[112] found FOXP1 positive MALT lymphomas to be at an increased risk of transforming into aggressive DLBCLs. Likewise, overexpression of FOXP1 in a subset of DLBCL indicates poor prognosis.[111, 112, 254, 255] The primary means responsible for FOXP1 deregulation has remained contentious due to the seldom occurrence of *FOXP1* gene rearrangements in FOXP1-positive lymphomas.[111, 256] As such, post-transcriptional regulation of FOXP1 by miR-34a is a conceivable alternative mechanism underlying the frequent deregulation of the transcription factor in lymphomas.

While the correlation data argue for FOXP1 having a possible role in the pathogenesis of DLBCL, experimental data is lacking to support a tumour promoting function. To address this issue we transiently knocked down FOXP1 activity by siRNA in DLBCL cell lines in order to monitor the effect on proliferation status. Indeed, the knockdown of FOXP1 recapitulated the blocked proliferation phenotype of DLBCL cells observed by enforced expression of miR-34a. The beneficial therapeutic effects of miR-34a are therefore anticipated due to its potent tumour suppressive properties exerted via the FOXP1 oncoprotein. Of future interest is to define the precise mechanism of FOXP1 activity in the context of haematological malignancies. At present, FOXP1 is known to be an essential regulator of normal early B-cell development and regulates immunoglobulin rearrangements by controlling the expression of both *RAG1* and *RAG2* genes.[109] As yet there have been no comprehensive studies to identify additional FOXP1 target genes which may aid in uncovering how this transcriptional repressor contributes to lymphomagenesis. Taken together, our results indicate that FOXP1 is frequently overexpressed in gDLBCL, is a direct target of MYC-dependent miR-34a, and represents a bona fide oncoprotein in this disease entity (Figure 4.2). We further propose miR-34a as a potential therapeutic target in the treatment of miR-34a-deficient, FOXP1-overexpressing lymphomas such as gDLBCL.

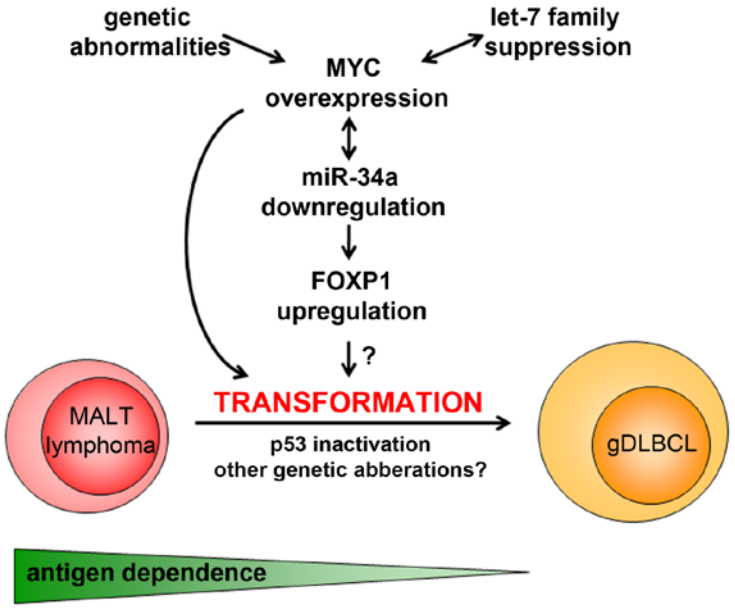


Figure 4.2 Hypothetical model of the progression of MALT lymphoma to gastric diffuse large B-cell lymphoma (gDLBCL). Aberrant expression of MYC can be caused by genetic abnormalities or by post-transcriptional regulation through miRNAs, such as let-7 family members. MYC overexpression and the resultant repression of miR-34a leads to FOXP1 deregulation and ultimate high grade transformation into gDLBCL as evidenced by loss of antigen dependence.

Gastric DLBCL is an aggressive fast-growing disease with inferior survival rates compared to MALT lymphoma. The addition of a chimeric anti-CD20 monoclonal antibody, rituximab, in combination with standard CHOP treatment has been shown to significantly increase survival in DLBCL patients.[257, 258] Despite this progress, there is a clear need for the development of more effective therapeutic strategies in order to improve the outlook for patients who have undergone transformation to DLBCL. Therapeutic strategies based on miRNA have added a new dimension to the treatment of cancer. miRNA replacement therapy is based on the concept of reintroducing a depleted, naturally occurring, tumour suppressive miRNA into cancer cells in order to suppress proliferation. In this regard, miRNA replacement therapy aims to restore a loss of function in tumour cells, unlike the inhibitory approach of other therapeutic modalities such as kinase inhibitors and siRNA. In a recent breakthrough study, Mendell and colleagues demonstrated in a mouse model of hepatocellular carcinoma that viral-mediated systemic delivery of a single miRNA, miR-26a, reversed disease progression without toxicity.[259] Most recently, the group of Bader used a non-viral lipid-based approach to systemically deliver synthetic miR-34a to block tumour growth in a mouse model of lung cancer.[260] In both studies, the treatment was well-tolerated, highly tumour specific and did not induce an immune response.

Together these findings provide proof-of-concept support for the systemic delivery of anti-tumourigenic miRNAs as a powerful therapeutic approach. Although the *in vivo* potential of miRNA replacement therapy has not yet been explored in the context of heamatopoietic cancers, manipulating miR-34a levels to suppress tumour cell proliferation presents an exciting and promising approach for the treatment of DLBCL. It should also be noted, that other types of lymphomas, such as CLL[261] and Burkitt's lymphoma,[262] are miR-34a deficient. This strengthens the rationale for exploring the therapeutic potential of miR-34a in malignancies beyond solid tumours. In particular, Burkitt's lymphoma, represents an ideal model to investigate the potential benefit of miR-34a replacement in MYC overexpressing lymphomas. The challenge of *in vivo* delivery to effectively target tumour B cells will be the chief focus of future therapeutic development efforts to harness the full potential of miR-34a as a tumour suppressor agent.

Taken together, our findings provide novel insights into the molecular detail of the complex biological mechanisms underpinning the unique pathogenesis of MALT lymphoma. Our studies reinforce this disease entity as an excellent model to study how antigenic drive, microenvironmental stimuli and epigenetic deregulation coalesce to promote lymphomagenesis and ultimately pave the way for the development and design of rational therapeutic modalities.

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6 CURRICULUM VITAE

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- Honours subject: Biochemistry; title of honours thesis: Exploring the Molecular Basis of the LIM Code
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PUBLICATIONS:

Craig VJ, Cogliatti SB, Rehrauer H, Wündisch T and Müller A. (2010) Epigenetic silencing of miRNA-203 dysregulates ABL1 expression and drives *Helicobacter*-associated gastric lymphomagenesis. *Manuscript submitted***.**

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PUBLICATIONS (continued):

Craig VJ, Arnold I, Gerke C, Huynh MQ, Wundisch T, Neubauer A, Renner C, Falkow S, Mueller A.(2010).Gastric MALT lymphoma B cells express polyreactive, somatically mutated immunoglobulins. *Blood*. 115(3):581-591.

Craig VJ, Cogliatti SB, Arnold I, Gerke C, Balandat JE, Wündisch T and Müller A. B-cell receptor signaling and CD40 ligand-independent T cell help cooperate in Helicobacter-induced MALT lymphomagenesis. *Leukemia* (2010) 24, 1186–1196

Matthews JM, Bhati M, **Craig VJ**, Deane JE, Lee C, Nancarrow AL, Ryan DP, Sunde M. (2008) Competition between LIM binding domains. *Biochem Soc Trans*. Dec 36;(6): 1393-7

Bhati M, Lee C, Nancarrow AL, Lee M, **Craig VJ**, Bach I, Guss JM, Mackay JP, Matthews JM. (2008) Implementing the LIM code: the structural basis for cell type-specific assembly of LIM-homeodomain complexes. *EMBO J*. Jul 23;27(14):2018-29.

PATENTS:

2010:

• Treatment of ABL overexpressing B-cell lymphoma

• Treatment of B-cell lymphoma with microRNA

GRANTS:

2009:

• Awarded URPP Research Project grant

"Investigation of microRNA regulation in MALT lymphomagensis"

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ACADEMIC AWARDS:

2006:

- Award of the University Medal based on academic merit
- Deans Honours List for academic excellence
- First Class Honours

2005:

- Gabriella Wittman Prize for Proficiency in Senior Genetics
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2004 :

- Deans Honours List for academic excellence
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